

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

NIPPON SHINYAKU CO., LTD.,)	
)	
Plaintiff,)	
)	
v.)	C.A. No. 21-1015 (GBW)
)	
SAREPTA THERAPEUTICS, INC.,)	VOLUME 2 (Part 1) (Exhibits 13-28)
)	
Defendant.)	

SAREPTA THERAPEUTICS, INC.,)	
)	
Defendant and Counter-Plaintiff,)	
)	
v.)	
)	
NIPPON SHINYAKU CO., LTD.)	
and NS PHARMA, INC.)	
)	
Plaintiff and Counter-)	
Defendants.)	

**JOINT APPENDIX TO CLAIM CONSTRUCTION BRIEF
FOR THE WILTON/UWA PATENTS**

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March 20, 2023

EXHIBIT 13

FDA NEWS RELEASE

FDA grants accelerated approval to first targeted treatment for rare Duchenne muscular dystrophy mutation

For Immediate Release:

December 12, 2019

The U.S. Food and Drug Administration today granted accelerated approval to Vyondys 53 (golodirsen) injection to treat Duchenne muscular dystrophy (DMD) patients who have a confirmed mutation of the dystrophin gene that is amenable to exon 53 skipping. It is estimated that about 8 percent of patients with DMD have this mutation.

“The FDA recognizes the urgent need for new medical treatments for serious neurological disorders and we have a long-standing commitment to working with researchers, drug companies and patients to facilitate the development and approval of treatments for rare diseases. With today’s accelerated approval, patients with Duchenne — a rare and devastating disease — who have a confirmed mutation of the dystrophin gene amenable to exon 53 skipping will now have available the first treatment targeted specifically for this disease subtype,” said Billy Dunn, M.D., acting director of the Office of Neuroscience in the FDA’s Center for Drug Evaluation and Research. “Use of the accelerated approval pathway will make Vyondys 53 available to patients based on initial data and we look forward to learning more about the drug’s clinical benefit from the ongoing confirmatory clinical trial.”

DMD is a rare genetic disorder characterized by progressive muscle deterioration and weakness. It is the most common type of muscular dystrophy (<https://www.ninds.nih.gov/Disorders/All-Disorders/Muscular-Dystrophy-Information-Page>). DMD is caused by an absence of dystrophin, a protein that helps keep muscle cells intact. The first symptoms are usually seen between three and five years of age and worsen over time. The disease often occurs in people without a known family history of the condition and primarily affects boys, but in rare cases it can affect girls. DMD occurs in about one out of every 3,600 male infants worldwide.

People with DMD progressively lose the ability to perform activities independently and often require a wheelchair by their early teens. As the disease progresses, life-threatening heart and respiratory conditions can occur. Patients typically succumb to the disease in their 20s or 30s; however, disease severity and life expectancy vary.

Vyondys 53 was approved under the accelerated approval pathway, which provides for the approval of drugs that treat serious or life-threatening diseases and generally offer a meaningful advantage over existing treatments. Approval under this pathway can be based on adequate and well-controlled studies showing the drug has an effect on a surrogate endpoint that is reasonably likely to predict clinical benefit to patients (i.e., how patients feel or function or whether they survive). This pathway provides earlier patient access to promising new drugs while the company conducts clinical trials to verify the predicted clinical benefit.

The accelerated approval of Vyondys 53 is based on the surrogate endpoint (/drugs/development-resources/surrogate-endpoint-resources-drug-and-biologic-development) of an increase in dystrophin production in the skeletal muscle observed in some patients treated with the drug. The FDA has concluded that the data submitted by the applicant demonstrated an increase in dystrophin production that is reasonably likely to predict clinical benefit in patients with DMD who have a confirmed mutation of the dystrophin gene amenable to exon 53 skipping. A clinical benefit of the drug, including improved motor function, has not been established. In making this decision, the FDA considered the potential risks associated with the drug, the life-threatening and debilitating nature of the disease and the lack of available therapy.

Vyondys 53 was evaluated in a two-part clinical study. The first part included 12 DMD patients, with eight patients receiving Vyondys 53 and four receiving placebo. The second part of the study was open-label, and included the 12 patients enrolled in part one of the study, and 13 additional patients who had not previously received the treatment. In the study, dystrophin levels increased, on average, from 0.10% of normal at baseline to 1.02% of normal after 48 weeks of treatment with the drug or longer.

As part of the accelerated approval process, the FDA is requiring the company to conduct a clinical trial to confirm the drug's clinical benefit. The ongoing study is designed to assess whether Vyondys 53 improves motor function of DMD patients with a confirmed mutation of the dystrophin gene amenable to exon 53 skipping. If the trial fails to verify clinical benefit, the FDA may initiate proceedings to withdraw approval of the drug.

The most common side effects reported by participants receiving Vyondys 53 in clinical studies were headache, fever (pyrexia), cough, vomiting, abdominal pain, cold symptoms (nasopharyngitis) and nausea. Hypersensitivity reactions, including rash, fever, itching, hives, skin irritation (dermatitis) and skin peeling (exfoliation), have occurred in patients who were treated with Vyondys 53.

Additionally, renal toxicity was observed in animals who received golodirsen. Although renal toxicity was not observed in the clinical studies with Vyondys 53, renal toxicity, including potentially fatal glomerulonephritis, has been observed after administration of some antisense oligonucleotides. Renal function should be monitored in patients taking Vyondys 53.

The FDA granted this application Fast Track (</patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/fast-track>) and Priority Review (</patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/priority-review>) designations. Vyondys 53 also received Orphan Drug (</industry/medical-products-rare-diseases-and-conditions/designating-orphan-product-drugs-and-biological-products>) designation, which provides incentives to assist and encourage the development of drugs for rare diseases. In addition, the manufacturer received a rare pediatric disease priority review voucher. The FDA's rare pediatric disease priority review voucher program (</drugs/development-resources/rare-pediatric-disease-priority-review-voucher-program-section-529>) is intended to encourage development of new drugs and biologics to prevent and treat rare diseases in children.

Approval of Vyondys 53 was granted to Sarepta Therapeutics of Cambridge, Massachusetts.

The FDA, an agency within the U.S. Department of Health and Human Services, protects the public health by assuring the safety, effectiveness, and security of human and veterinary drugs, vaccines and other biological products for human use, and medical devices. The agency also is responsible for the safety and security of our nation's food supply, cosmetics, dietary supplements, products that give off electronic radiation, and for regulating tobacco products.

Related Information

- National Institute of Neurological Disorders and Stroke: Muscular Dystrophy Information (<https://www.ninds.nih.gov/Disorders/All-Disorders/Muscular-Dystrophy-Information-Page>).
- FDA: Approved Drugs: Questions and Answers (</drugs/information-consumers-and-patients-drugs/approved-drugs-questions-and-answers>).
- FDA: New Drugs at FDA (</drugs/development-approval-process-drugs/new-drugs-fda-cders-new-molecular-entities-and-new-therapeutic-biological-products>).

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EXHIBIT 14

FDA NEWS RELEASE

FDA Approves Targeted Treatment for Rare Duchenne Muscular Dystrophy Mutation

For Immediate Release:

August 12, 2020

Today, the U.S. Food and Drug Administration granted accelerated approval to Viltepso (viltolarsen) injection for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the DMD gene that is amenable to exon 53 skipping. This is the second FDA-approved targeted treatment for patients with this type of mutation. Approximately 8% of patients with DMD have a mutation that is amenable to exon 53 skipping.

“The FDA is committed to fostering drug development for serious neurological disorders like Duchenne muscular dystrophy,” said Billy Dunn, M.D., director of the Office of Neuroscience in the FDA’s Center for Drug Evaluation and Research. “Today’s approval of Viltepso provides an important treatment option for Duchenne muscular dystrophy patients with this confirmed mutation.”

DMD is a rare genetic disorder characterized by progressive muscle deterioration and weakness. It is the most common type of muscular dystrophy (<https://www.ninds.nih.gov/Disorders/All-Disorders/Muscular-Dystrophy-Information-Page>). DMD is caused by mutations in the DMD (<https://ghr.nlm.nih.gov/gene/DMD>) gene that results in an absence of dystrophin, a protein that helps keep muscle cells intact. The first symptoms are usually seen between three and five years of age and worsen over time. DMD occurs in approximately one out of every 3,600 male infants worldwide; in rare cases, it can affect females.

Viltepso was evaluated in two clinical studies with a total of 32 patients, all of whom were male and had genetically confirmed DMD. The increase in dystrophin production was established in one of those two studies, a study that included 16 DMD patients, with 8 patients receiving Viltepso at the recommended dose. In the study, dystrophin levels increased, on average, from 0.6% of normal at baseline to 5.9% of normal at week 25.

The FDA concluded that the applicant’s data demonstrated an increase in dystrophin production that is reasonably likely to predict clinical benefit in patients with DMD who have a confirmed mutation of the dystrophin gene amenable to exon 53 skipping. A clinical benefit of

the drug has not been established. In making this decision, the FDA considered the potential risks associated with the drug, the life-threatening and debilitating nature of the disease, and the lack of available therapies.

As part of the accelerated approval (</patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/accelerated-approval>) process, the FDA is requiring the company to conduct a clinical trial to confirm the drug's clinical benefit. The ongoing study is designed to assess whether Viltepso improves the time to stand for DMD patients with this confirmed mutation. If the trial fails to verify clinical benefit, the FDA may initiate proceedings to withdraw approval of the drug.

The most common side effects observed in DMD patients (pooled from the two studies) treated with 80 mg/kg once a week were: Upper respiratory tract infection, injection site reaction, cough and fever.

Although kidney toxicity was not observed in the Viltepso clinical studies, the clinical experience with Viltepso is limited, and kidney toxicity, including potentially fatal glomerulonephritis, has been observed after administration of some antisense oligonucleotides. Kidney function should be monitored in patients taking Viltepso.

Viltepso was approved under the FDA's accelerated approval pathway, which provides for the approval of drugs that treat serious or life-threatening diseases and generally offer a meaningful advantage over existing treatments. Approval under this pathway can be based on adequate and well-controlled studies showing the drug has an effect on a surrogate endpoint that is reasonably likely to predict clinical benefit to patients (i.e., how patients feel or function or whether they survive). This pathway provides earlier patient access to promising new drugs while the company conducts clinical trials to verify the predicted clinical benefit.

The FDA granted this application Priority Review (</patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/priority-review>) designation.

The FDA is granting the approval to NS Pharma, Inc.

The FDA, an agency within the U.S. Department of Health and Human Services, protects the public health by assuring the safety, effectiveness, and security of human and veterinary drugs, vaccines and other biological products for human use, and medical devices. The agency also is responsible for the safety and security of our nation's food supply, cosmetics, dietary supplements, products that give off electronic radiation, and for regulating tobacco products.

Related Information

- [NIH: Duchenne muscular dystrophy](https://rarediseases.info.nih.gov/diseases/6291/duchenne-muscular-dystrophy)
(<https://rarediseases.info.nih.gov/diseases/6291/duchenne-muscular-dystrophy>).
- [Accelerated Approval](/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/accelerated-approval) (/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/accelerated-approval).
- [Priority Review](/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/priority-review) (/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/priority-review).

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EXHIBIT 15

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VYONDYS 53 safely and effectively. See full prescribing information for VYONDYS 53.

VYONDYS 53 (golodirsen) injection, for intravenous use
Initial U.S. Approval: 2019

RECENT MAJOR CHANGES

Dosage and Administration (2.1, 2.2, 2.3, 2.4)	2/2021
Warnings and Precautions (5.2)	2/2021

INDICATIONS AND USAGE

VYONDYS 53 is an antisense oligonucleotide indicated for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the *DMD* gene that is amenable to exon 53 skipping. This indication is approved under accelerated approval based on an increase in dystrophin production in skeletal muscle observed in patients treated with VYONDYS 53. Continued approval for this indication may be contingent upon verification of a clinical benefit in confirmatory trials. (1)

DOSAGE AND ADMINISTRATION

- Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VYONDYS 53 (2.1)
- 30 milligrams per kilogram once weekly (2.2)
- Administer as an intravenous infusion over 35 to 60 minutes via an in-line 0.2 micron filter (2.2, 2.4)
- Dilution required prior to administration (2.3)

DOSAGE FORMS AND STRENGTHS

Injection: 100 mg/2 mL (50 mg/mL) in a single-dose vial (3)

CONTRAINDICATIONS

None (4)

WARNINGS AND PRECAUTIONS

- Hypersensitivity Reactions:** Hypersensitivity reactions, including rash, pyrexia, pruritus, urticaria, dermatitis, and skin exfoliation have occurred in patients who were treated with VYONDYS 53. If a hypersensitivity reaction occurs, institute appropriate medical treatment and consider slowing the infusion or interrupting the VYONDYS 53 therapy. (2.3, 5.1)
- Kidney Toxicity:** Based on animal data, may cause kidney toxicity. Kidney function should be monitored; creatinine may not be a reliable measure of renal function in DMD patients. (5.2, 13.2)

ADVERSE REACTIONS

The most common adverse reactions (incidence $\geq 20\%$ and higher than placebo) were headache, pyrexia, fall, abdominal pain, nasopharyngitis, cough, vomiting, and nausea. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact Sarepta Therapeutics, Inc. at 1-888-SAREPTA (1-888-727-3782) or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

See 17 for PATIENT COUNSELING INFORMATION

Revised: 2/2021

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FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

VYONDYS 53 is indicated for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the *DMD* gene that is amenable to exon 53 skipping. This indication is approved under accelerated approval based on an increase in dystrophin production in skeletal muscle observed in patients treated with VYONDYS 53 [see *Clinical Studies* (14)]. Continued approval for this indication may be contingent upon verification of a clinical benefit in confirmatory trials.

2 DOSAGE AND ADMINISTRATION

2.1 Monitoring to Assess Safety

Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VYONDYS 53. Consider measurement of glomerular filtration rate prior to initiation of VYONDYS 53. Monitoring for kidney toxicity during treatment is recommended. Obtain the urine samples prior to infusion of VYONDYS 53 or at least 48 hours after the most recent infusion [see *Warnings and Precautions* (5.2)].

2.2 Dosing Information

The recommended dosage of VYONDYS 53 is 30 milligrams per kilogram administered once weekly as a 35 to 60-minute intravenous infusion via an in-line 0.2 micron filter.

If a dose of VYONDYS 53 is missed, it may be administered as soon as possible after the scheduled dose.

2.3 Preparation Instructions

VYONDYS 53 is supplied in single-dose vials as a preservative-free concentrated solution that requires dilution prior to administration. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. Use aseptic technique.

- a. Calculate the total dose of VYONDYS 53 to be administered based on the patient's weight and the recommended dose of 30 milligrams per kilogram. Determine the volume of VYONDYS 53 needed and the correct number of vials to supply the full calculated dose.
- b. Allow the vials to warm to room temperature. Mix the contents of each vial by gently inverting 2 or 3 times. Do not shake.
- c. Visually inspect each vial of VYONDYS 53. The solution is a clear to slightly opalescent, colorless liquid, and may contain trace amounts of small, white to off-white amorphous particles. Do not use if the solution in the vials is cloudy, discolored or

contains extraneous particulate matter other than trace amounts of small, white to off-white amorphous particles.

- d. With a syringe fitted with a 21-gauge or smaller bore non-coring needle, withdraw the calculated volume of VYONDYS 53 from the appropriate number of vials.
- e. Dilute the withdrawn VYONDYS 53 in 0.9% Sodium Chloride Injection, USP, to make a total volume of 100 to 150 mL. Gently invert 2 to 3 times to mix. Do not shake. Visually inspect the diluted solution. Do not use if the solution is cloudy, discolored or contains extraneous particulate matter other than trace amounts of small, white to off-white amorphous particles.
- f. Administer the diluted solution via an in-line 0.2 micron filter.
- g. VYONDYS 53 contains no preservatives and should be administered immediately after dilution. Complete infusion of diluted VYONDYS 53 within 4 hours of dilution. If immediate use is not possible, the diluted product may be stored for up to 24 hours at 2°C to 8°C (36°F to 46°F). Do not freeze. Discard unused VYONDYS 53.

2.4 Administration Instructions

Application of a topical anesthetic cream to the infusion site prior to administration of VYONDYS 53 may be considered.

VYONDYS 53 is administered via intravenous infusion. Flush the intravenous access line with 0.9% Sodium Chloride Injection, USP, prior to and after infusion.

Infuse the diluted VYONDYS 53 over 35 to 60 minutes via an in-line 0.2 micron filter. Do not mix other medications with VYONDYS 53 or infuse other medications concomitantly via the same intravenous access line with VYONDYS 53.

If a hypersensitivity reaction occurs, consider slowing the infusion or interrupting the VYONDYS 53 therapy [see *Warnings and Precautions (5.1) and Adverse Reactions (6.1)*].

3 DOSAGE FORMS AND STRENGTHS

VYONDYS 53 is a clear to slightly opalescent, colorless liquid, and may contain trace amounts of small, white to off-white amorphous particles, and available as:

- Injection: 100 mg/2 mL (50 mg/mL) solution in a single-dose vial

4 CONTRAINDICATIONS

None.

5 WARNINGS AND PRECAUTIONS

5.1 Hypersensitivity Reactions

Hypersensitivity reactions, including rash, pyrexia, pruritus, urticaria, dermatitis, and skin exfoliation have occurred in VYONDYS 53-treated patients, some requiring treatment. If a hypersensitivity reaction occurs, institute appropriate medical treatment and consider slowing the infusion or interrupting the VYONDYS 53 therapy [see *Dosage and Administration* (2.4)].

5.2 Kidney Toxicity

Kidney toxicity was observed in animals who received golodirsen [see *Use in Specific Populations* (8.4)]. Although kidney toxicity was not observed in the clinical studies with VYONDYS 53, the clinical experience with VYONDYS 53 is limited, and kidney toxicity, including potentially fatal glomerulonephritis, has been observed after administration of some antisense oligonucleotides. Kidney function should be monitored in patients taking VYONDYS 53. Because of the effect of reduced skeletal muscle mass on creatinine measurements, creatinine may not be a reliable measure of kidney function in DMD patients. Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VYONDYS 53. Consider also measuring glomerular filtration rate using an exogenous filtration marker before starting VYONDYS 53. During treatment, monitor urine dipstick every month, and serum cystatin C and urine protein-to-creatinine ratio every three months. Only urine expected to be free of excreted VYONDYS 53 should be used for monitoring of urine protein. Urine obtained on the day of VYONDYS 53 infusion prior to the infusion, or urine obtained at least 48 hours after the most recent infusion, may be used. Alternatively, use a laboratory test that does not use the reagent pyrogallol red, as this reagent has the potential to cross react with any VYONDYS 53 that is excreted in the urine and thus lead to a false positive result for urine protein.

If a persistent increase in serum cystatin C or proteinuria is detected, refer to a pediatric nephrologist for further evaluation.

6 ADVERSE REACTIONS

The following serious adverse reactions are described below and elsewhere in the labeling:

- Hypersensitivity Reactions [see *Warnings and Precautions* (5.1)]

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

In the VYONDYS 53 clinical development program, 58 patients received at least one intravenous dose of VYONDYS 53, ranging between 4 mg/kg (0.13 times the recommended dosage) and 30 mg/kg (the recommended dosage). All patients were male and had genetically confirmed Duchenne muscular dystrophy. Age at study entry was 6 to 13 years. Most (86%) patients were Caucasian.

VYONDYS 53 was studied in 2 double-blind, placebo-controlled studies.

In Study 1 Part 1, patients were randomized to receive once-weekly intravenous infusions of VYONDYS 53 (n=8) in four increasing dose levels from 4 mg/kg to 30 mg/kg or placebo (n=4), for at least 2 weeks at each level. All patients who participated in Study 1 Part 1 (n=12) were continued into Study 1 Part 2, an open-label extension, during which they received VYONDYS 53 at a dose of 30 mg/kg IV once weekly [see *Clinical Studies (14)*].

In Study 2, patients received VYONDYS 53 (n=33) 30 mg/kg or placebo (n=17) IV once weekly for up to 96 weeks, after which all patients received VYONDYS 53 at a dose of 30 mg/kg.

Adverse reactions observed in at least 20% of treated patients in the placebo-controlled sections of Studies 1 and 2 are shown in [Table 1](#).

Table 1: Adverse Reactions That Occurred in At Least 20% of VYONDYS 53-Treated Patients and at a Rate Greater than Placebo in Studies 1 and 2

Adverse Reaction	VYONDYS 53 (N = 41) %	Placebo (N = 21) %
Headache	41	10
Pyrexia	41	14
Fall	29	19
Abdominal pain	27	10
Nasopharyngitis	27	14
Cough	27	19
Vomiting	27	19
Nausea	20	10

Other adverse reactions that occurred at a frequency greater than 5% of VYONDYS 53-treated patients and at a greater frequency than placebo were: administration site pain, back pain, pain, diarrhea, dizziness, ligament sprain, contusion, influenza, oropharyngeal pain, rhinitis, skin abrasion, ear infection, seasonal allergy, tachycardia, catheter site related reaction, constipation, and fracture.

Hypersensitivity reactions have occurred in patients treated with VYONDYS 53 [see *Warnings and Precautions (5.1)*].

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

There are no human or animal data available to assess the use of VYONDYS 53 during pregnancy. In the U.S. general population, major birth defects occur in 2 to 4% and miscarriage occurs in 15 to 20% of clinically recognized pregnancies.

8.2 Lactation

Risk Summary

There are no human or animal data to assess the effect of VYONDYS 53 on milk production, the presence of golodirsen in milk, or the effects of VYONDYS 53 on the breastfed infant.

The developmental and health benefits of breastfeeding should be considered along with the mother's clinical need for VYONDYS 53 and any potential adverse effects on the breastfed infant from VYONDYS 53 or from the underlying maternal condition.

8.4 Pediatric Use

VYONDYS 53 is indicated for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the *DMD* gene that is amenable to exon 53 skipping, including pediatric patients [*see Clinical Studies (14)*].

Intravenous administration of golodirsen (0, 100, 300, or 900 mg/kg) to juvenile male rats once weekly for 10 weeks (postnatal days 14 to 77) did not result in postnatal developmental (e.g., neurobehavioral, immune function, or male reproductive) toxicity. However, at the highest dose tested (900 mg/kg/week), golodirsen resulted in the death of animals because of renal impairment or failure. In surviving animals (including one animal at the lowest dose tested), there was a dose-dependent increase in the incidence and severity of renal tubular effects (including degeneration/regeneration, fibrosis, vacuolation, and dilatation), which correlated with changes in clinical pathology parameters, reflecting a dose-dependent impairment of renal function. In addition, decreases in bone area, mineral content, and mineral density were observed at the highest dose tested (900 mg/kg week) but with no effect on bone growth. A no-effect dose for renal toxicity was not identified; the lowest dose tested (100 mg/kg/week) was associated with plasma exposures (AUC) approximately 2.5 times that in humans at the recommended human dose of 30 mg/kg/week.

8.5 Geriatric Use

DMD is largely a disease of children and young adults; therefore, there is no geriatric experience with VYONDYS 53.

8.6 Patients with Renal Impairment

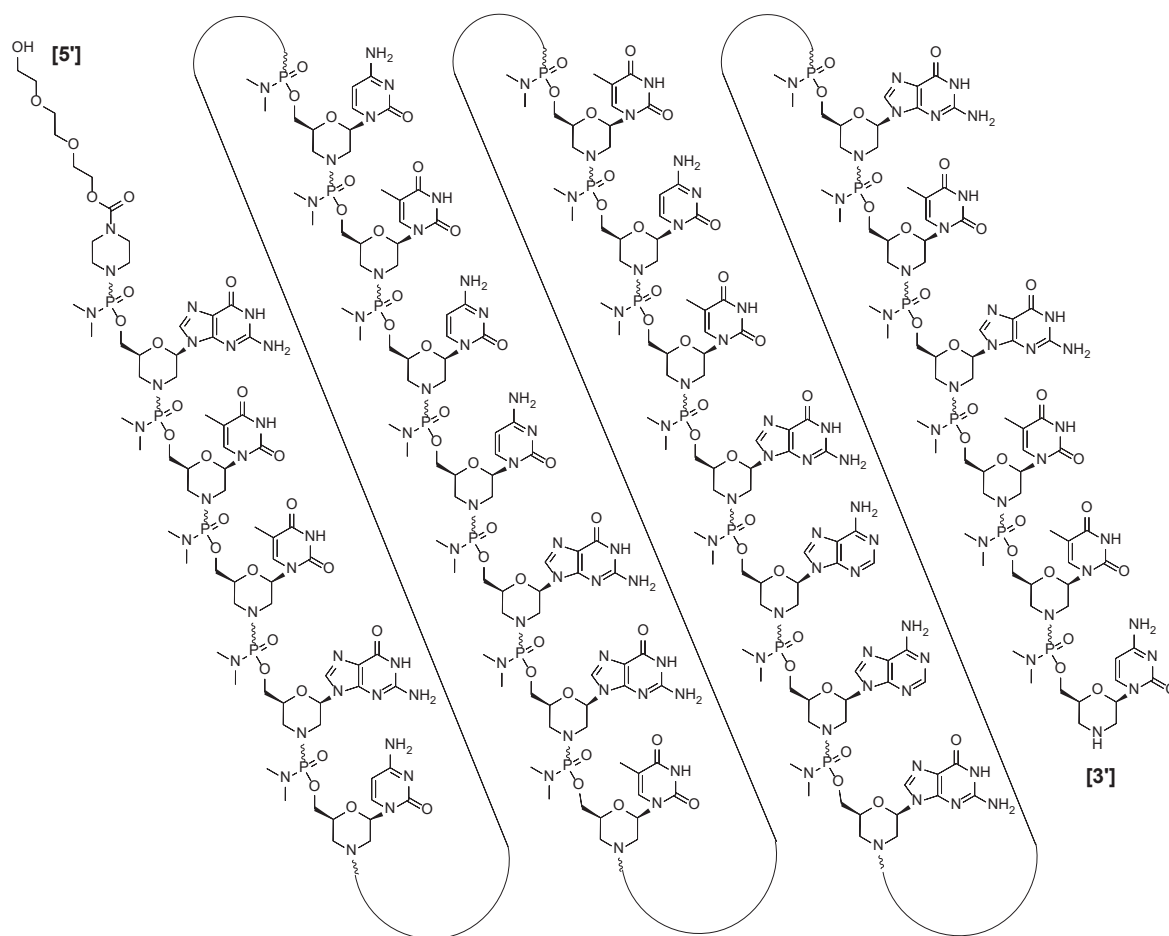
Renal clearance of golodirsen is reduced in non-DMD adults with renal impairment, based on estimated glomerular filtration rate calculated using the Modification of Diet and Renal Disease (MDRD) equation [see *Clinical Pharmacology* (12.3)]. However, because of the effect of reduced skeletal muscle mass on creatinine measurements in DMD patients, no specific dosage adjustment can be recommended for DMD patients with renal impairment based on estimated glomerular filtration rate. Patients with known renal function impairment should be closely monitored during treatment with VYONDYS 53.

11 DESCRIPTION

VYONDYS 53 (golodirsen) injection is a sterile, aqueous, preservative-free, concentrated solution for dilution prior to intravenous administration. VYONDYS 53 is a clear to slightly opalescent, colorless liquid, and may contain trace amounts of small, white to off-white amorphous particles. VYONDYS 53 is supplied in single-dose vials containing 100 mg golodirsen (50 mg/mL). VYONDYS 53 is formulated as an isotonic phosphate buffered saline solution with an osmolality of 260 to 320 mOSM and a pH of 7.5. Each milliliter of VYONDYS 53 contains: 50 mg golodirsen; 0.2 mg potassium chloride; 0.2 mg potassium phosphate monobasic; 8 mg sodium chloride; and 1.14 mg sodium phosphate dibasic, anhydrous, in water for injection. The product may contain hydrochloric acid or sodium hydroxide to adjust pH.

Golodirsen is an antisense oligonucleotide of the phosphorodiamidate morpholino oligomer (PMO) subclass. PMOs are synthetic molecules in which the five-membered ribofuranosyl rings found in natural DNA and RNA are replaced by a six-membered morpholino ring. Each morpholino ring is linked through an uncharged phosphorodiamidate moiety rather than the negatively charged phosphate linkage that is present in natural DNA and RNA. Each phosphorodiamidate morpholino subunit contains one of the heterocyclic bases found in DNA (adenine, cytosine, guanine, or thymine). Golodirsen contains 25 linked subunits. The sequence of bases from the 5' end to 3' end is GTTGCCTCCGGTTCTGAAGGTGTTC. The molecular formula of golodirsen is $C_{305}H_{481}N_{138}O_{112}P_{25}$ and the molecular weight is 8647.28 daltons.

The structure of golodirsen is:



12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Golodirsen is designed to bind to exon 53 of dystrophin pre-mRNA resulting in exclusion of this exon during mRNA processing in patients with genetic mutations that are amenable to exon 53 skipping. Exon 53 skipping is intended to allow for production of an internally truncated dystrophin protein in patients with genetic mutations that are amenable to exon 53 skipping [see *Clinical Studies* (14)].

12.2 Pharmacodynamics

After treatment with VYONDYS 53, all patients evaluated (n=25) in Study 1 Part 2 [see *Clinical Studies* (14)] had an increase in skipping of exon 53 demonstrated by reverse transcription polymerase chain reaction (RT-PCR), compared to baseline.

In Study 1 Part 2 [see *Clinical Studies* (14)], dystrophin levels as assessed by the Sarepta western blot assay increased from 0.10% (SD 0.07) of normal at baseline to 1.02% (SD 1.03) of normal after 48 weeks of treatment with VYONDYS 53. The mean change from baseline in

dystrophin after 48 weeks of treatment with VYONDYS 53 was 0.92% (SD 1.01) of normal levels ($p < 0.001$); the median change from baseline was 0.88%. This increase in dystrophin protein expression positively correlated with the level of exon skipping. Dystrophin levels assessed by western blot can be meaningfully influenced by differences in sample processing, analytical technique, reference materials, and quantitation methodologies. Therefore, comparing dystrophin results from different assay protocols will require a standardized reference material and additional bridging studies.

Correct localization of truncated dystrophin to the sarcolemma in muscle fibers of patients treated with golodirsen was demonstrated by immunofluorescence staining.

12.3 Pharmacokinetics

The pharmacokinetics of golodirsen was evaluated in DMD patients following administration of intravenous doses ranging from 4 mg/kg/week to 30 mg/kg/week (i.e., recommended dosage). Golodirsen exposure increased proportionally with dose, with minimal accumulation with once-weekly dosing. Inter-subject variability (as %CV) for C_{\max} and AUC ranged from 38% to 72%, and 34% to 44%, respectively.

Distribution

Steady-state volume of distribution was similar between DMD patients and healthy subjects. The mean golodirsen steady-state volume of distribution was 668 mL/kg (%CV=32.3) at a dose of 30 mg/kg. Golodirsen plasma protein binding ranged from 33% to 39% and is not concentration dependent.

Elimination

Golodirsen elimination half-life (SD) was 3.4 (0.6) hours, and plasma clearance was 346 mL/hr/kg at the 30 mg/kg dose.

Metabolism

Golodirsen is metabolically stable. No metabolites were detected in plasma or urine.

Excretion

Golodirsen is mostly excreted unchanged in the urine. The elimination half-life ($t_{1/2}$) was 3.4 hours.

Specific Populations

Age:

The pharmacokinetics of golodirsen have been evaluated in male pediatric DMD patients. There is no experience with the use of VYONDYS 53 in DMD patients 65 years of age or older.

Sex:

Sex effects have not been evaluated; VYONDYS 53 has not been studied in female patients.

Race:

The potential impact of race is not known because 92% of the patients in studies were Caucasians.

Patients with Renal Impairment:

The effect of renal impairment on the pharmacokinetics of golodirsen was evaluated in non-DMD subjects aged 41 to 65 years with Stage 2 chronic kidney disease (CKD) (n=8, estimated glomerular filtration rate (eGFR) ≥ 60 and < 90 mL/min/1.73 m²) or Stage 3 CKD (n=8, eGFR ≥ 30 and < 60 mL/min/1.73 m²) and matched healthy subjects (n=8, eGFR ≥ 90 mL/min/1.73 m²). Subjects received a single 30 mg/kg IV dose of golodirsen.

In subjects with Stage 2 or Stage 3 CKD, exposure (AUC) increased approximately 1.2-fold and 1.9-fold, respectively. There was no change in the C_{max} in subjects with Stage 2 CKD; in subjects with Stage 3 CKD, there was a 1.2-fold increase in C_{max} compared with subjects with normal renal function. The effect of Stage 4 or Stage 5 CKD on golodirsen pharmacokinetics and safety has not been studied.

Estimated GFR values derived from MDRD equations and the threshold definitions for various CKD stages in otherwise healthy adults would not be generalizable to pediatric patients with DMD. Therefore, no specific dosage adjustment can be recommended for patients with renal impairment [see *Use in Specific Populations* (8.6)].

Patients with Hepatic Impairment:

VYONDYS 53 has not been studied in patients with hepatic impairment.

Drug Interaction Studies

Golodirsen did not inhibit CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP3A4/5 *in vitro*. Golodirsen was a weak inducer of CYP1A2 and did not induce CYP2B6 or CYP3A4. Golodirsen was not metabolized by human hepatic microsomes and was not a substrate or strong inhibitor of any of the key human drug transporters tested (OAT1, OAT3, OCT2, OATP1B1, MATE1, P-gp, BCRP, and MRP2, OATP1B3 and MATE2-K). Based on *in vitro* data, golodirsen has a low potential for drug-drug interactions in humans.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenesis

Carcinogenicity studies have not been conducted with golodirsen.

Mutagenesis

Golodirsen was negative in *in vitro* (bacterial reverse mutation and chromosomal aberration in CHO cells) and *in vivo* (mouse bone marrow micronucleus) assays.

Impairment of Fertility

Fertility studies in animals were not conducted with golodirsen. No effects of golodirsen on the male reproductive system were observed following weekly subcutaneous administration (0, 120,

300, or 600 mg/kg to male mice or weekly intravenous administration (0, 80, 200, or 400 mg/kg) to male monkeys. Plasma exposure (AUC) at the highest doses tested in mouse and monkey are approximately 10 and 45 times that in humans at the recommended weekly intravenous dose of 30 mg/kg.

13.2 Animal Toxicology and/or Pharmacology

Kidney toxicity was observed in studies in male mice and rats; findings in urinary bladder were observed in male mice.

In male mice, golodirsén was administered weekly for 12 weeks by intravenous injection (0, 12, 120, or 960 mg/kg) or for 26 weeks by subcutaneous injection (0, 120, 300, or 600 mg/kg). In the 12-week study, microscopic findings in kidney (tubular dilatation, basophilic or eosinophilic casts, vacuolation), correlated with increases in serum markers of renal function (e.g., urea nitrogen, creatinine), were observed primarily at the highest dose tested; hypertrophy of the transitional epithelium of the ureter or urinary bladder was observed at all doses. In the 26-week study, renal tubular degeneration and degeneration of the transitional epithelium of the urinary bladder were observed at all doses.

In male rats, intravenous administration of golodirsén (0, 60, 100, 300, or 600 mg/kg) weekly for 13 weeks resulted in tubular degeneration at all but the lowest dose tested; at the high dose, the microscopic changes were accompanied by increases in serum urea nitrogen.

In male monkeys, intravenous administration of golodirsén (0, 80, 200, or 400 mg/kg) weekly for 39 weeks resulted in microscopic changes in kidney (basophilia, dilatation, or mononuclear cell infiltration) at all doses, which correlated with increases in serum markers of renal function (urea nitrogen, creatinine) at the highest dose tested.

14 CLINICAL STUDIES

The effect of VYONDYS 53 on dystrophin production was evaluated in one study in DMD patients with a confirmed mutation of the *DMD* gene that is amenable to exon 53 skipping (Study 1; NCT02310906).

Study 1 Part 1 was a double-blind, placebo-controlled, dose-titration study in 12 DMD patients. Patients were randomized 2:1 to receive VYONDYS 53 or matching placebo. VYONDYS 53-treated patients received four escalating dose levels, ranging from 4 mg/kg/week (less than the recommended dosage) to 30 mg/kg/week, by intravenous infusion for 2 weeks at each dose level.

Study 1 Part 2 was a 168-week, open-label study assessing the efficacy and safety of VYONDYS 53 at a dose of 30 mg/kg/week in the 12 patients enrolled in Part 1, plus 13 additional treatment-naïve patients with DMD amenable to exon 53 skipping. At study entry (either in Part 1 or Part 2), patients had a median age of 8 years and were on a stable dose of corticosteroids for at least 6 months. Efficacy was assessed based on change from baseline in the dystrophin protein level (measured as % of the dystrophin level in healthy subjects, i.e., % of normal) at Week 48 of Part 2. Muscle biopsies were obtained at baseline prior to treatment and at Week 48 of Part 2 in all VYONDYS 53-treated patients (n=25), and were analyzed for dystrophin protein level by Sarepta western blot. Mean dystrophin levels increased from 0.10% (SD 0.07) of normal at

baseline to 1.02% (SD 1.03) of normal by Week 48 of Study 1 Part 2, with a mean change in dystrophin of 0.92% (SD 1.01) of normal levels ($p < 0.001$); the median change from baseline was 0.88%.

Individual patient dystrophin levels from Study 1 are shown in [Table 2](#).

Table 2: Dystrophin Expression Sarepta Western Blot by Individual Patient From Study 1

Patient Number	Sarepta Western Blot % Normal Dystrophin			Patient number	Sarepta Western Blot % Normal Dystrophin		
	Baseline	Part 2 Week 48	Change from baseline		Baseline	Part 2 Week 48	Change from baseline
1	0.08	0.09	0.01	14	0.22	0.28	0.06
2	0.11	0.11	0.01	15	0.14	0.21	0.07
3	0.21	0.22	0.01	16	0.05	0.42	0.37
4	0.05	0.12	0.08	17	0.07	1.03	0.97
5	0.03	0.12	0.09	18	0.02	1.57	1.55
6	0.06	0.14	0.09	19	0.12	1.17	1.05
7	0.12	0.37	0.25	20	0.03	1.72	1.69
8	0.11	1.06	0.95	21	0.11	1.77	1.66
9	0.06	0.54	0.48	22	0.31	4.30	3.99
10	0.05	0.97	0.92	23	0.11	0.36	0.25
11	0.06	1.55	1.49	24	0.03	0.91	0.88
12	0.07	1.91	1.84	25	0.07	1.29	1.22
13	0.10	3.25	3.15				

16 HOW SUPPLIED/STORAGE AND HANDLING

16.1 How Supplied

VYONDYS 53 injection is supplied in single dose vials. The solution is a clear to slightly opalescent, colorless liquid, and may contain trace amounts of small, white to off-white amorphous particles.

- Single-dose vials containing 100 mg/2mL (50 mg/mL) NDC 60923-465-02

16.2 Storage and Handling

Store VYONDYS 53 at 2°C to 8°C (36°F to 46°F). Do not freeze. Store in original carton until ready for use to protect from light.

17 PATIENT COUNSELING INFORMATION

Hypersensitivity Reactions

Advise patients and/or caregivers that hypersensitivity reactions, including rash, pyrexia, pruritus, urticaria, dermatitis, and skin exfoliation have occurred in patients who were treated with VYONDYS 53. Instruct them to seek immediate medical care should they experience signs and symptoms of hypersensitivity [*see Warnings and Precautions (5.1)*].

Kidney Toxicity

Inform patients nephrotoxicity has occurred with drugs similar to VYONDYS 53. Advise patients of the importance of monitoring for kidney toxicity by their healthcare providers during treatment with VYONDYS 53 [*see Warnings and Precautions (5.2)*].

Manufactured for:
Sarepta Therapeutics, Inc.
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EXHIBIT 16

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Conservation of the Duchenne Muscular Dystrophy Gene in Mice and Humans

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A portion of the Duchenne muscular dystrophy (DMD) gene transcript from human fetal skeletal muscle and mouse adult heart was sequenced, representing approximately 25 percent of the total, 14-kb DMD transcript. The nucleic acid and predicted amino acid sequences from the two species are nearly 90 percent homologous. The amino acid sequence that is predicted from this portion of the DMD gene indicates that the protein product might serve a structural role in muscle, but the abundance and tissue distribution of the messenger RNA suggests that the DMD protein is not nebulin.

DUCHENNE MUSCULAR DYSTROPHY (DMD) is an X-linked, recessive, human genetic disorder that affects approximately 1 in every 3500 males in all populations that have been studied (1). Clinical onset of Duchenne muscular dystrophy is first observed in affected children when they are about 2 to 3 years old, the first evidence being proximal muscle weakness. Biochemical onset of this disease occurs much earlier as seen by histological examination of affected fetal muscle (2). Target tissue differences are also observed with regard to clinical versus histological phenotype. Specifically, heart function is only minimally affected, even though heart muscle appears very similar histologically to affected skeletal muscle (3). The rapid and progressive wasting of striated muscle, characteristic of DMD, leads to death by the end of the second decade.

Despite nearly 100 years of intensive study, little is known about the primary biochemical defect responsible for the destruction of muscle in individuals with DMD. We have previously described the isolation of genomic DNA that is closely linked to the DMD gene and the delineation of two small putative exons of the DMD gene within the cloned genomic regions (4-7). One of these exons was used to isolate a 1-kb segment of human fetal skeletal muscle

complementary DNA (cDNA) (FSM 5-1) that spans a portion of the Xp21 DMD locus (6). We now report the isolation and characterization of cDNAs corresponding to the homologous locus in the mouse, a comparison of the human and mouse DMD gene expression patterns, and the DNA and predicted protein sequence for about 25% of the total DMD transcript.

Previous work on the identification of human DMD coding sequences was based primarily on the high degree of conservation

of two small putative exons from mouse and man (6). Since the expression pattern of the 14-kb human DMD gene transcript (8) had been studied only in fetal tissues, and in view of the difficulties in obtaining large amounts of various human tissues, we extended our transcriptional studies by using the human cDNA as a probe for Northern blots of mouse tissue RNA (9). The human cDNA detected a 14-kb mouse mRNA species, which was present in very low levels in tissue extracts of mouse newborn leg and gravid (15-day) combined uterus and placenta and in higher levels in newborn heart, adult heart, and adult skeletal muscle (Fig. 1). The abundance of the mDMD (mouse Duchenne muscular dystrophy) transcript is roughly one-thousandth that of the mouse α -tubulin transcript (Fig. 1). We detected DMD transcription in all of the striated muscle that we tested except for fetal mouse tissues containing skeletal muscle. Our inability to detect fetal transcription was most likely due to the heterogeneity of the fetal mouse tissues, the lack of differentiation in the muscle fibers at early stages of mouse development, or both (10).

The smooth muscle cell layers of gravid mammalian uteri undergo massive hypertrophy in preparation for parturition. The gravid uteri of E15-bearing mice (3 days before parturition) are, therefore, an excellent source of mitotically and metabolically active smooth muscle. Our finding of a very low level of mouse DMD gene transcription in E15 combined uterus and placenta is

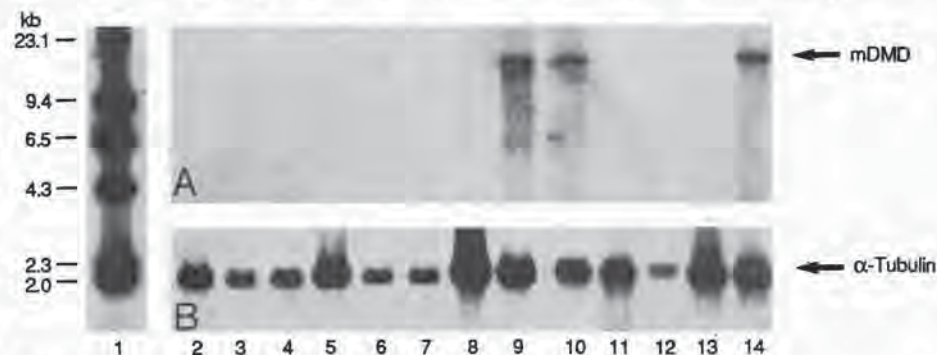


Fig. 1. Expression of the mouse DMD locus. Polyadenylated RNA (7 μ g) was separated by electrophoresis in a 1% agarose-formaldehyde gel. The fragments were transferred to a nylon membrane and hybridized with a 32 P-labeled (23) human fetal skeletal muscle cDNA, corresponding to the human DMD locus (7, 9). The 14-kb mRNA species, corresponding to the mouse DMD gene is shown. As a control for the amount and efficacy of the RNA that was loaded, the nylon membranes were dehybridized and rehybridized with a labeled cDNA clone, corresponding to the mouse α -tubulin gene. The 2-kb mRNA, corresponding to α -tubulin, is shown for each lane. In (A) (mDMD) autoradiographic exposure was for 10 days; in (B) (tubulin) exposure was for 2 hours, that is, one-hundredth the time used for (A). All autoradiography was done with an intensifying screen at -80°C , and each probe was labeled to similar specific activities. The same blots used with the human cDNA probe were then dehybridized and again probed with the mouse cDNA, MC2-6. The results with the mouse probe were identical to those with the human probe. Lanes are as follows: 1, λ DNA Hind III-digest markers; 2, E13 (13-day-old embryos); 3, E13 uterus and placenta; 4, E15 carcass; 5, E15 brain; 6, E15 uterus and placenta; 7, E15 viscera; 8, newborn brain; 9, newborn heart and lung; 10, adult heart; 11, adult kidney; 12, adult liver; 13, adult lung; 14, adult skeletal muscle. Additional tissues that were tested but not shown were as follows: mouse gravid uteri of embryonic day 5 (E5), E7, and E9; isolated mouse embryos of E10 and E11; adult brain, spleen, small intestine, stomach, and testes. All of these tissues tested negative for the mDMD transcript.

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most likely due to expression in the abundant smooth muscle tissue. Thus, the expression pattern of the human DMD gene in mouse is identical to, and otherwise complements, the expression pattern of the human DMD in fetal tissues (7, 11).

In order to obtain cDNAs representative of the mouse DMD gene, a cDNA library was constructed from adult mouse heart (12) and screened with the partial cDNA corresponding to the human DMD gene (FSM-5). Seven mouse cDNA clones were obtained from 5×10^5 primary (unamplified) λ gt10 recombinants. Six of these were encompassed within the largest 2.7-kb cDNA, MC2-6, corresponding to the sequences shown 5' to the Eco RI site in Fig. 2. The additional 1.7-kb of cardiac cDNA sequences, 3' of the Eco RI site, were isolated from the same cDNA library by screening with a mouse skeletal muscle cDNA clone that had been isolated from an adult skeletal muscle cDNA library. All of the clones that have been isolated from the mouse skeletal muscle library to date are

identical to those isolated from the mouse cardiac muscle library. In this second screening of the cardiac muscle library ten identical clones were obtained.

The low frequency of DMD clones obtained in both human (6) and mouse cDNA libraries suggests that the DMD messenger RNA (mRNA) is rare, probably representing about 0.01 to 0.001% of the mRNA in muscle. This relative abundance is in agreement with our Northern blot analyses and the frequency with which recombinant DMD clones were obtained from four other mouse and human cDNA libraries constructed in our laboratory.

Fragments of the mouse cDNA were then used as probes for Southern blots of mouse genomic DNA. At least 12 X chromosome-specific, Hind III-digested DNA fragments were detected by the 5' fragment (2.7 kb) of cDNA sequences (MC2-6). Ten are evident in Fig. 2 (13). Since there was only one internal Hind III site in the mouse cDNA clone, this 2.7-kb clone hybridized to a minimum of 11 regions distributed over at

least 90 kb of the mouse X chromosome (13). In view of the 14-kb size of the complete mRNA, and if we assume a constant ratio of cDNA to genomic DNA, the complete mouse DMD genomic locus probably encompasses more than 500 kb of genomic DNA. Thus, the hybridization characteristics of the X-linked human DMD (7, 8) are also conserved in the mouse.

The human fetal skeletal muscle cDNA library, originally used to obtain the 1-kb FSM 5-1 cDNA (6), was completely digested at Eco RI sites because methylation did not occur at those sites during construction of the cDNA library. Since the 3' terminus of FSM 5-1 contained an endogenous Eco RI site rather than an added linker, we used a genomic exon fragment spanning the Eco RI site as a probe to obtain cDNAs on the 3' side of this Eco RI site. In this manner we obtained a 1.2-kb cDNA fragment that was contiguous with that described previously. Additional Hind III-digested, X chromosome-specific, genomic DNA fragments, were detected by this cDNA (Fig. 2).

The DNA sequences of the human and mouse DMD cDNAs were determined throughout the region of overlap (2.2 kb), with the mouse sequence extending approximately 500 bp both 5' and 3' of the overlap (Fig. 3). The total nucleotide sequence of 3.3 kb represents nearly 25% of the entire DMD mRNA and contains one continuous open reading frame. Both the DNA and amino acid sequences are well conserved, exhibiting 88% homology with the DNA and 87% with the amino acid sequence. There is a particularly striking conservation in the hydropathicity profile of the mouse and human amino acid sequences (14), and the hydropathicity profiles are nearly identical (Fig. 4). Indeed, if conservative amino acid substitutions are permitted based on hydropathicity values, the mouse and human polypeptides become more than 95% homologous.

The protein sequence that is predicted from this portion of the DMD gene has a number of interesting characteristics. Chou and Fasman calculations predict a strong propensity for an α -helical secondary structure over the entire 100 kD of protein sequence (14). Locally, charged residues alternate with hydrophobic residues in a manner resembling those found in tropomyosin and the myosin rod, both which contain hydrophobic residues concentrated at two positions within a heptad repeat. This creates a hydrophobic interface between neighboring α -helical coils, resulting in a coiled-coil (15). In fact, NBRF protein database searches found homologies to many such α helix-rich, coiled-coil proteins (14). For example, regions of more than 100 amino

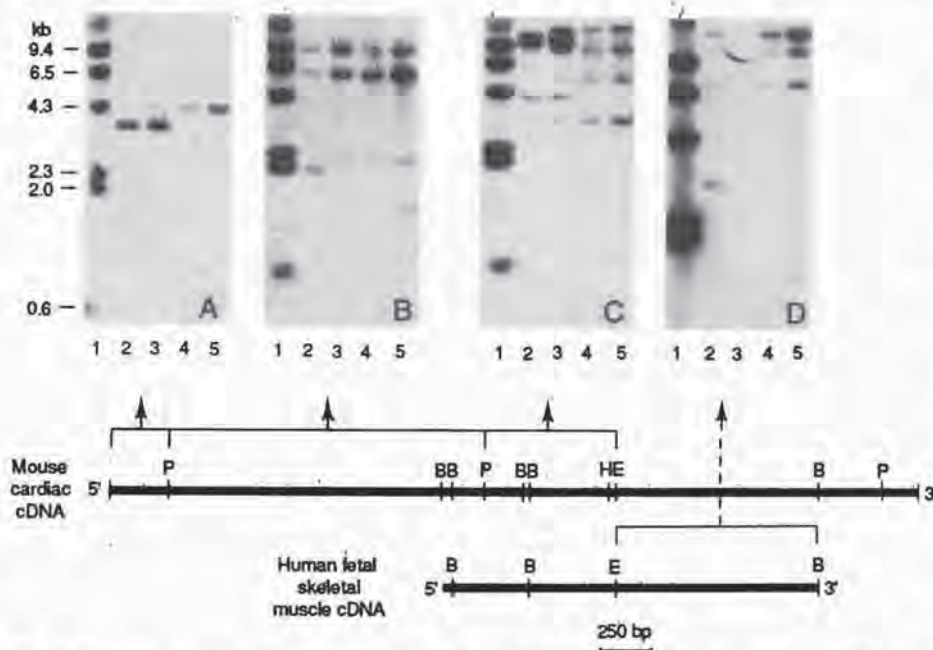


Fig. 2. Restriction maps and genomic analyses of partial DMD cDNAs in mouse and man. This is an extension of a previously described human fetal skeletal muscle DMD cDNA segment [FSM-5; (7)] and the overlapping mouse adult heart cDNA segments. The human cDNA library has been described (7). The mouse adult heart cDNA library was constructed in λ gt10 (13). DMD-homologous sequences from both human and mouse recombinant phage were subcloned into plasmid vectors (Bluescript, Stratagene) and used as probes against Southern blots of genomic DNA (23, 24). A total of 10 of the 12 mouse genomic Hind III fragments recognized by the mouse cDNA, MC2-6 (A, B, and C) (13), are shown in addition to the human genomic Hind III fragments recognized by the extension of the human cDNA (D). Because of the high degree of conservation of this locus (7), all of the cDNA clones exhibited an X chromosome-specific pattern and recognized genomic fragments in the hamster DNA backgrounds. Lanes for panels A, B, and C are the following Hind III-digested genomic DNA samples: 1, labeled (32 P) λ DNA Hind III digest markers; 2, hamster; 3, hamster somatic cell-hybrid bearing a mouse X chromosome; 4, male mouse; and 5, female mouse. Panel D has the following Hind III-digested DNA samples: 1, radiolabeled λ DNA Hind III digest markers; 2, hamster hybrid cell line containing a human X chromosome; 3, lymphoblastoid cell line from a patient having an Xp21 deletion (25); 4, normal male, 46,XY; 5, 49,XXXXY cell line (GM1202). Restriction enzyme sites are as follows: P, Pst I; E, Eco RI; H, Hind III; and B, Bgl II.

Fig. 3. Nucleotide and predicted amino acid sequences of mouse and human cDNAs for a portion of the DMD gene. The cDNAs shown in Fig. 2 were subcloned in plasmid vectors (Stratagene Bluescript) and subjected to either chemical (26) or chain terminating (27) sequencing methods. Clones were sequenced on both strands. Sequence translations and alignments were done on the BIONET resource, Intelligene. Differences between the mouse and human DNA sequences are indicated by asterisks, while amino acid differences are boxed.

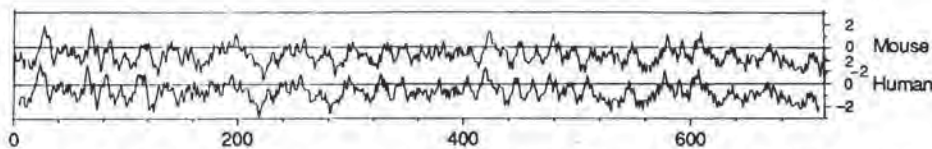
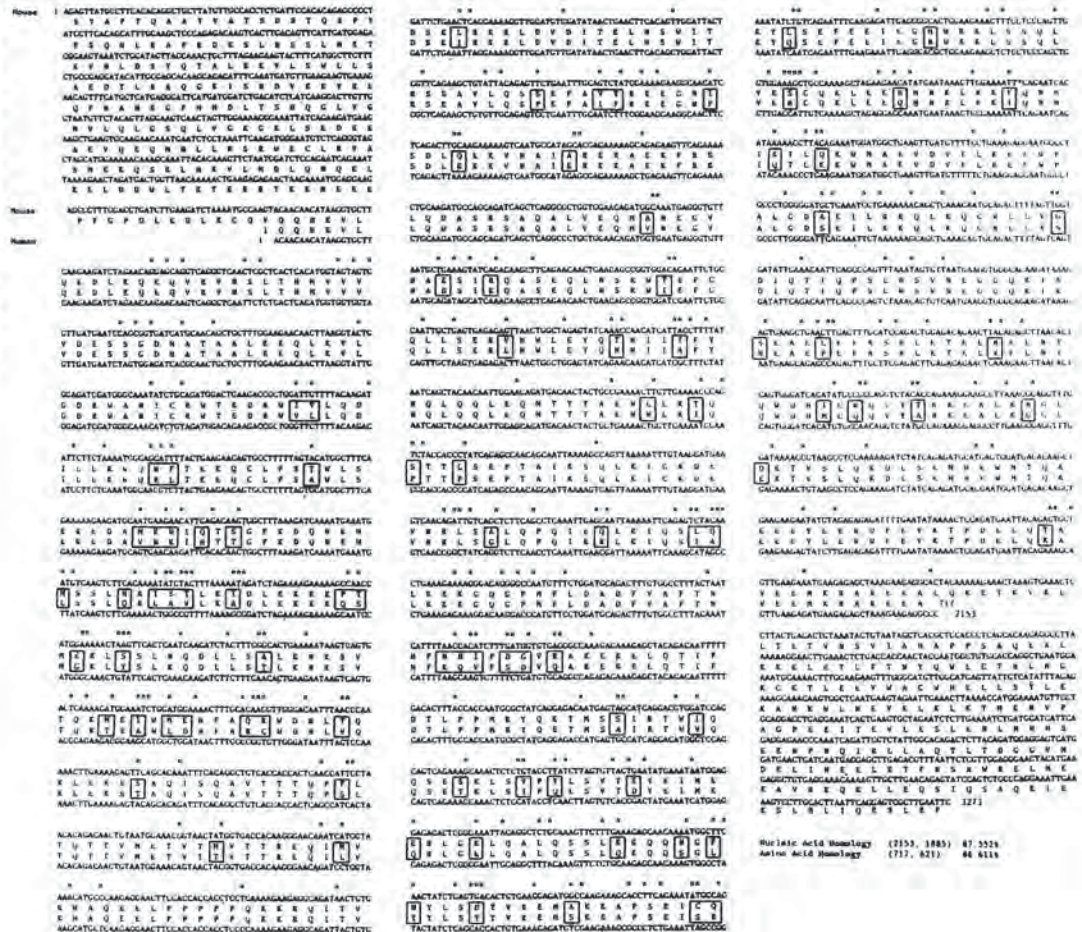


Fig. 4. Hydropathicity profiles of sequenced regions of the DMD cDNAs. Hydropathicity plots, obtained with Kyte and Doolittle calculations (14), were done at the Howard Hughes Medical Institute Computing Center, Harvard Medical School, with the use of default values of the University of Wisconsin's PEPPILOT program. The horizontal axis shows the amino acid residue number of the human sequence; the vertical axis gives the relative hydropathicity of each plot.

acids of the DMD protein are 20% homologous to the myosin rod of both nematode and rat (14). Unlike these perfect coiled-coils, however, this section of the DMD protein contains occasional helix-breaking residues, suggesting that it might adopt an α -helical bundle conformation, rather than an extended rod structure (16). In addition, the initial 650 residues exhibit a fairly constant ratio of charged to apolar amino acids of 0.7, whereas extended rod structural proteins usually have ratios from 0.9 to 1.4 (16). In the NH_2 -terminal fragment, containing 440 residues, the ratio of charged to apolar amino acids is 1.0, and there are fewer prolines and a stricter adherence to the heptad repeat.

The high degree of homology in the sequence of this protein from mouse and

man suggests either that it interacts with a number of other highly conserved structural proteins or it serves a specific structural role in muscle tissue. The low ratio of charged to apolar amino acid residues in the NH_2 -terminal region suggests that the protein product of the DMD gene might be associated peripherally or integrally with the muscle fiber plasma membrane (sarcolemma). However, the primary amino acid sequence is also reminiscent of most sarcomeric proteins, such that a structural role for the DMD product within the sarcomere is also possible.

A large sarcomeric protein, nebulin, has been proposed as a candidate for the DMD gene product due to its large size (500 kD) and its apparent absence in the muscle of affected individuals (17). Nebulin has been

estimated to represent roughly 3% of total myofibrillar protein (18). We have calculated that 0.01 to 0.001% of total cardiac and skeletal muscle mRNA is DMD mRNA. Although it is difficult to equate the abundance of a particular protein to the abundance of its corresponding mRNA, this large difference between the amounts of nebulin protein and DMD mRNA seems significant. Comparative studies of the distribution of nebulin throughout the animal kingdom have revealed an analogue of nebulin in the skeletal muscle of most organisms but not in any cardiac muscle (rabbit, sheep, cow, rat, and bullfrog) (19, 20). We have shown that the DMD gene is expressed equally well in skeletal and cardiac muscle in both humans and mice (Fig. 1). The differences in tissue distribution and abundance between the DMD mRNA and the nebulin protein suggest that nebulin is probably not the primary product of the DMD gene. However, the disturbed patterns of nebulin protein homeostasis observed in DMD-affected individuals implicate nebulin as a potentially important component in the etiology of this disease.

The prominent abnormality in boys affected with DMD is the loss of skeletal muscle. Also associated with DMD are car-

diac abnormalities (3, 21) and, less often, mental retardation (22). The transcriptional data that have been presented for the DMD gene thus far account for the skeletal and cardiac muscle defects, but do not explain the mental retardation. When more is understood about the DMD and surrounding Xp21 loci, the mental retardation seen in some DMD boys may be explained. Further insights into the basic biochemical defect in DMD must await specific protein studies with antibodies directed against the DMD protein product, analyses of the specific mutations giving rise to the DMD phenotype, and cell-based expression studies.

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8. The size of the DMD transcript has been lowered to 14 kb due to our recent cloning of the entire DMD human cDNA [M. Koenig et al., *Cell* **50**, 509 (1987)].
9. RNA was isolated from tissues by homogenization of frozen, ground tissues in guanidium thiocyanate, followed by pelleting through a CsCl cushion [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)]. Polyadenylated RNA [H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972)] was separated by electrophoresis in 1% agarose formaldehyde gels and then transferred to nylon membranes. Hybridization was performed as recommended by the manufacturer [Biodyne A, New England Nuclear; P. S. Thomas, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5201 (1980)].
10. C. A. Maltin, L. Duncan, A. B. Wilson, *Muscle Nerve* **8**, 211 (1984); V. Dubowitz, *Nature (London)* **197**, 1215 (1963).
11. The number and positions of the RNA samples on the Northern blots that were reported previously (6) were incorrectly assigned. The human DMD gene transcription attributed to expression in small intestine and lung was instead due to expression in human fetal heart. Later experiments have shown that the expected 14-kb human DMD transcript is present in fetal heart at levels equivalent to those found in fetal skeletal muscle.
12. The cDNA library was constructed using a modification of the oligo(dT) primed, ribonuclease H procedure of U. Gubler and B. J. Hoffman [*Gene* **25**, 263 (1983)], with modifications suggested by Stratagene's librarian, H. Short, including a Sepharose 4B exclusion column to eliminate Eco RI linkers and cDNA molecules shorter than 600 bp. We used the vector λ gt10 [T. V. Huynh, R. A. Young, R. W. Davis, in *DNA Cloning*, D. M. Glover, Ed. (IRL Press, Washington, DC, 1985), vol. 1, pp. 49-78].
13. Mouse genomic locus size was calculated as follows: mouse genomic DNA, digested with Hind III, was fractionated on a 10-cm, 0.7% agarose gel, transferred to nitrocellulose (24) and hybridized with five overlapping subfragments of MC2-6 (Fig. 2). Two additional, weakly hybridizing Hind III fragments were observed but are not evident in this figure. The single-copy genomic fragments detected by the five probes are 22 kb, 12 kb, 11 kb, 8.5 kb, 8 kb, 5.9 kb, 5 kb, 4.9 kb, 4.3 kb, 3 kb, 2.1 kb, and 1.1 kb. These fragments total more than 90 kb and represent a minimum estimate of the genomic size as there are probably many more Hind III genomic DNA fragments lying between those detected by our probes (6).
14. Hydropathy measurements and plots, protein secondary structure predictions, NBRF (National Biomedical Research Foundation) protein sequence database searches, and lowered stringency homology searches between myosin and the DMD protein were done on the BIONET (Palo Alto, CA) or the Howard Hughes Medical Institute (Harvard Medical School, Boston, MA) computer systems. Both the University of Wisconsin Genetics Computer Group (U-WISC) and Intelligenetics (BIONET) software packages were used. α helix- β sheet propensities were calculated according to P. Y. Chou and G. D. Fasman [*Biochemistry* **13**, 222 (1974)]. Hydropathy measurements were calculated according to Kyte and Doolittle [*J. Mol. Biol.* **157**, 105 (1982)]. Figure 4 shows the hydropathy plot output of the U-WISC PEPLOT program.
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27. F. Sanger, S. Nicklen, A. R. Coulson, *ibid.*, p. 5463.
28. We thank H. Short for her cDNA library protocol, S. P. Kwan and S. Whitehead for the generous synthesis of oligonucleotides, D. Shine for his gift of a mouse α -tubulin cDNA clone, C. Disteché and R. Farber for their gift of the hamster somatic cell hybrid bearing a mouse X chromosome, R. Neve for supplying a human fetal Northern blot and skeletal muscle cDNA library, and J. Schwaber, S. Latt, and C. Cohen for helpful criticisms of the manuscript. E.H. is the Harry Zimmerman Post-doctoral Fellow of the Muscular Dystrophy Association. A.P.M. is supported by the Muscular Dystrophy Association and PHS NRSA (2T 32 GM07753-07) from the National Institute for General Medical Sciences. Supported by grants to L.M.K. from the Muscular Dystrophy Association and the National Institutes of Health (R01 NS23740 and HD18658). L.M.K. is an associate investigator of the Howard Hughes Medical Institute.

20 May 1987; accepted 24 July 1987

Depolarization Without Calcium Can Release γ -Aminobutyric Acid from a Retinal Neuron

ERIC A. SCHWARTZ

Calcium influx is often an essential intermediate step for the release of neurotransmitter. However, some retinal neurons appear to release transmitter by a mechanism that does not require calcium influx. It was uncertain whether depolarization released calcium from an intracellular store or released transmitter by a mechanism that does not require calcium. The possibility that voltage, and not calcium, can regulate the release of transmitter was studied with pairs of solitary retinal neurons. Horizontal and bipolar cells were isolated from fish retinas and juxtaposed in culture. Communication between them was studied with electrophysiological methods. A horizontal cell released its neurotransmitter, γ -aminobutyric acid, when depolarized during conditions that buffered the internal calcium concentration and prohibited calcium entry. The speed and amount of material released were sufficient for a contribution to synaptic transmission.

CALCIUM-TRIGGERED EXOCYTOSIS operates to release transmitters at neuromuscular junctions, between peripheral neurons, and in the brain. Synapses that utilize this mechanism are often recognized by two features: first, an aggregation of vesicles marks each presynaptic site and second, Ca^{2+} is required for transmitter release. Exceptions to this ubiquitous mechanism may now have been identified in the distal retina (1, 2). Studies of its anatomy and physiology indicate that both photoreceptors and horizontal cells make synapses that operate differently.

Photoreceptors make two morphological types of synapse (3): one has an aggregation

of vesicles at a release site, the other is vesicle poor. Isolated photoreceptors release transmitters by two mechanisms (2): one requires extracellular Ca^{2+} , the other continues in the absence of Ca^{2+} . Moreover, one component of the normal synaptic transmission from photoreceptors to postsynaptic cells requires extracellular Ca^{2+} (4), while another component functions without extracellular Ca^{2+} (5).

Complementary observations have been made for horizontal cells. They make synapses that lack vesicles (6) and release the

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Conservation of the Duchenne Muscular Dystrophy Gene in Mice and Humans

Eric P. Hoffman, Anthony P. Monaco, Chris C. Feener, and Louis M. Kunkel

Science, 238 (4825), • DOI: 10.1126/science.3659917

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EXHIBIT 17

BoxInterferences@uspto.gov

Entered: May 12, 2016

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT TRIAL AND APPEAL BOARD

University of Western Australia,
Junior Party
(Patent 8,455,636,
Inventors: Stephen Donald Wilton, Sue Fletcher and Graham McClorey),

v.

Academisch Ziekenhuis Leiden,
Senior Party
(Application 11/233,495,
Inventors: Garrit-Jan Boudewijn van Ommen, Judith Christina
Theodora van Deutekom, Johannes Theodorus den Dunnen and
Annemieke Aartsma-Rus).

Patent Interference No. 106,007 (RES)
(Technology Center 1600)

Before: RICHARD E. SCHAFER, SALLY GARDNER LANE, and DEBORAH
KATZ, *Administrative Patent Judges*

SCHAFER, *Administrative Patent Judge.*

Decision - Motions - 37 CFR § 41.125(a) (Substitute)

- 1 This interference is between University of Western Australia (UWA)
- 2 Patent 8,455,636 and Academisch Ziekenhuis Leiden (AZL)
- 3 Application 11/233,495.

I.

The parties have presented the following motions for decision:

(1) UWA Motion 4 to exclude certain AZL evidence. Paper 455

(2) UWA Motion 1 asserting that AZL's claims are unpatentable under 35 U.S.C. § 112(a) because the claims are broader than supported by the written description and/or an enabling disclosure. Paper 210.

(3) UWA Motion 2 asserting that AZL's claims are unpatentable under 35 U.S.C. § 112(b) as indefinite. Paper 211.

(4) UWA Motion 3 seeking the declaration of an additional interference between UWA U.S. Patent No. 8,455,636 AZL Application No. 14/248,279. Paper 212.

(5) AZL Motion 1 asserting that certain of UWA claims are unpatentable over prior art. Paper 181

(6) AZL Motion 2 to deny UWA the accorded benefit date of is Australian Application AU 2004903474. Paper 26.

(7) AZL Motion 3 asserting that certain of UWA's claims are unpatentable under 35 U.S.C. § 101 in view of Association for *Molecular Pathology v. Myriad Genetics, Inc.*, 133 S.Ct. 2107 (2013). Paper 27.

(8) AZL Motion 4 (Responsive) to add two additional claims to its application. Paper 241.

II.

The involved subject matter

The subject matter claimed by the parties relates to "exon skipping." Exon skipping is a molecular biology technique that may be useful for ameliorating or eliminating the effects of certain genetic mutations. Those mutations may result in a shift in the reading frame during protein formation resulting in a non-functional

1 or partially functional protein. The exon skipping technique, in effect, hides
2 certain pre-mRNA exons from the mRNA formation machinery. As a result, the
3 hidden exon is removed along with introns during the splicing to form mRNA.
4 The exon skipping is said to be caused by the binding of an oligonucleotide that
5 includes a nucleobase sequence that is complementary to a portion of a particular
6 pre-mRNA exon. The complimentary oligonucleotide is referred to as an antisense
7 oligonucleotide or “AON.” Both the exon to be discarded and the AON are chosen
8 to restore an open reading frame allowing for the formation of a more complete
9 and more functional protein.

10 Specifically, the parties’ invention is directed to AONs selected to cause
11 skipping of exon 53 of the pre-mRNA associated with the gene responsible for the
12 formation of the protein dystrophin. The absence of dystrophin prevents skeletal
13 muscle development and causes the myopathies of muscular dystrophy. In people
14 suffering from Duchenne muscular dystrophy (DMD), the mutation in the
15 dystrophin gene essentially precludes the formation of any functional dystrophin.
16 By skipping, and thus removing, exon 53 during the formation of mRNA, a
17 reading frame is said to be restored, resulting in the formation of a partially
18 functional dystrophin protein.

19 III.

20 *UWA Motion 4 to exclude certain testimony*

21 UWA moves to exclude the testimony of Dr. Erik Sontheimer (Exs. 1012,
22 1067 and 1186) and certain portions of the testimony of Dr. van Deutekom
23 (Ex. 1125). We dismiss the motion.

24 With respect to Dr. Sontheimer, UWA challenges the admissibility of all
25 three of his declarations on the basis that he is not qualified as an expert in the field
26 under Fed. R. Evid. 702.

1 The prerequisite to filing a miscellaneous motion to exclude evidence is the
2 timely service of objections to that evidence on the opponent. SO ¶ 151.
3 Objections to evidence must be served within five business days of service of the
4 evidence. 37 C.F.R. § 41.155(b)(1). Where an objection to evidence is served, the
5 proponent of the evidence has ten business days to file supplemental evidence.
6 37 C.F.R. § 41.155(b)(2). The purpose of supplemental evidence is to allow the
7 proponent an opportunity to cure the alleged evidentiary defect. SO ¶ 155.3. If an
8 objection is not timely made, the objection is waived. SO ¶ 155.1.2. A motion to
9 exclude evidence must identify where in the record the objection was originally
10 made. 37 C.F.R. § 41.155(c); SO ¶ 155.2.2.

11 AZL filed three declarations by Dr. Sontheimer—Exhibits 1012, 1067 and
12 1186—on November 18, 2014, December 23, 2014, and February 17, 2015,
13 respectively. As the basis for its motion to exclude, UWA identifies its objections,
14 served February 24, 2015 (Ex. 2150). UWA Motion 4, Paper 455, 1:7-10. UWA
15 never objected to the first and second Sontheimer declarations (Exs. 1012 and
16 1067). Ex. 2150. Thus, the objections as to those declarations were untimely and
17 considered to have been waived. 37 C.F.R. § 41.155(b)(1); SO ¶ 155.1.2.

18 With respect to the third Sontheimer declaration (Ex. 1186), the objection on
19 its face appears to have been timely. However, we fail to see why the objection to
20 Dr. Sontheimer's expertise could not have been made in response to the filing of
21 his first declaration and CV (Ex. 1013). It is apparent from Dr. Sontheimer's
22 cross-examination, that UWA had significant questions as to his expertise that
23 could have formed the basis for a timely objection. The failure to timely give
24 notice of its objections precluded AZL from timely filing supplemental evidence
25 with respect to at least the first two Sontheimer declarations and possibly following
26 a modified course with respect to the third. Under these circumstances, we decline
27 to consider the exclusion of Dr. Sontheimer's third declaration. However, in

1 evaluating Dr. Sontheimer's testimony, we will consider UWA's arguments on his
2 expertise in evaluating the weight to be give his testimony.

3 UWA also seeks to exclude ¶¶ 4-18 of Dr. van Deutekom's testimony
4 (Ex. 1125). That testimony is directed to Dr. Sontheimer's credentials. UWA
5 Motion 4, Paper 455, 1:5-6. UWA posits two bases for exclusion: (1) under Fed.
6 R. Evid. 403, the value of her testimony is substantially outweighed by unfair
7 prejudice and needless presentation of cumulative evidence and (2) as hearsay
8 under Fed. R. Evid.. 801 and 802.

9 With respect to the first basis, the Board is fully capable of weighing any
10 probative value of her testimony against any unduly prejudicial effect.
11 Additionally, her testimony on the point, in light of the extensive record developed
12 in this proceeding, is not so extensive that it is excessively cumulative.

13 With respect to hearsay challenge, UWA did not raise hearsay as a basis to
14 object to Dr. van Deutekom's testimony in its objections. Ex. 2150. Thus, its
15 objection to admissibility on this ground is considered untimely.

16 UWA's motion to exclude is dismissed as to Exhibits 1012, 1067 and 1186
17 and denied with respect paragraphs 4 to 18 of Exhibit 1125.

18 IV.

19 *UWA Motion 1 - Unpatentability under 35 U.S.C. § 112(a)*

20 A.

21 UWA Motion 1 argues that AZL's involved claims are unpatentable because
22 AZL's written description is insufficient to support the full scope of subject matter
23 claimed. More specifically, UWA argues that AON "operative sequences are
24 actually highly unpredictable, varying with parameters such as nucleobase
25 sequence, length, backbone chemistry, and internucleotide linkages." UWA
26 Motion 1, Paper 210, 1:14-16. Because we find that, at the time AZL filed its
27 involved application, the sequence length of AONs that would maintain exon

1 skipping was substantially unpredictable, we hold that AZL’s written description
2 did not reasonably convey possession of the full scope of the subject matter of
3 AZL’s Claims 15, 76, 78-80, 82, 84, 86, 88-90, 97, 98, and 100-103. We also hold
4 that AZL’s sole remaining claim—Claim 77—which is limited to the AON
5 designated h53AON1 having the nucleotide sequence designated by SEQ. ID NO:
6 29, has not been shown to lack an adequate written description.

7 **1.**

8 AZL’s claims are directed to AONs that cause exon 53 skipping during the
9 splicing of human dystrophin pre-mRNA. AZL Clean Copy of Claims, Paper 8.
10 AONs may be said to have two parts—the backbone and the nucleobases. The
11 backbone is the structural framework to which the nucleobases attach.
12 Conventional RNA has a phosphodiester-ribose backbone. Each of AZL’s claims
13 require that the AON backbone comprise “a modification selected from the group
14 consisting of” six or seven listed components or classes of components. In other
15 words at least some of the conventional RNA phosphodiester-ribose backbone
16 must be replaced by one or more of the recited components. AZL’s claims identify
17 seven possible modifications: 2’-O-methyl, 2’-O-methyl-phosphorothioate
18 (2OMePS), a morpholine ring, a phosphorodiamidate linkage, a modification to
19 increase resistance to RNaseH, a peptide nucleic acid and a locked nucleic acid.
20 *See, e.g.* Claim 78, AZL Clean Copy of Claims, Paper 8, 1:18-2:2.

21 At the center of AZL’s claimed subject matter is the AON whose nucleobase
22 sequence corresponds to AZL’s SEQ ID NO: 29 (cuguugccuccgguucug). AZL’s
23 disclosure identifies h53AON1 as an AON having that sequence. It also has a
24 2OMePS backbone. Ex. 1008. 26:14-21. That backbone has two substitutions
25 when compared to the conventional RNA backbone: (1) a phosphorothioate
26 linkage for the phosphodiester linkage and (2) 2’-O-methyl for the 2’-OH of the
27 ribose. h53AON1 is the sole AON identified in AZL’s disclosure to cause exon 53

1 skipping. Ex. 1008, p. 48. That AON is 18 nucleobases in length. Ex. 1008, p.
2 48, Table 2. The majority of AZL's claims, however, encompass AONs of up to
3 50 or 80 nucleobases.

4 We reproduce AZL's Claims 15, 77 and 78 below with paragraphing and
5 bracketed matter added (AZL Clean Copy of Claims, Paper 8):

6 AZL Claim 15.

7 An isolated antisense oligonucleotide of
8 [1] 15 to 80 nucleotides comprising
9 [a] at least 15 bases of the sequence
10 cuguugccuccgguucug (SEQ ID NO: 29),
11 [2] wherein said oligonucleotide induces exon 53
12 skipping in the human dystrophin pre-mRNA,
13 [3] said oligonucleotide comprising a modification
14 selected from the group consisting of:
15 [a] 2'-O-methyl,
16 [b] 2'-O-methyl-phosphorothioate,
17 [c] a morpholine ring,
18 [d] a phosphorodiamidate linkage,
19 [e] a peptide nucleic acid and
20 [f] a locked nucleic acid.

21
22 AZL Claim 77.

23 The oligonucleotide of claim 15, wherein the oligonucleotide is
24 [1] 18 nucleotides and comprises
25 [a] the base sequence of the sequence
26 cuguugccuccgguucug (SEQ ID NO: 29),
27 [2] wherein said oligonucleotide induces exon 53
28 skipping in the human dystrophin pre-mRNA.

29
30 AZL Claim 78.

31 An isolated antisense oligonucleotide of
32 [1] 18 to 50 nucleotides in length,
33 [a] wherein said oligonucleotide is
34 [i] capable of binding to an exon-internal
35 sequence of exon 53 of the human dystrophin
36 pre-mRNA and
37 [ii] inducing skipping of exon 53, and

[b] wherein h53AON1 (cuguugccuccgguucug)
(SEQ ID NO: 29) is capable of binding to said
exon-internal sequence of exon 53 pre-mRNA,
[2] said oligonucleotide comprising a modification
selected from the group consisting of:
[a] 2'-O-methyl,
[b] 2'-O-methyl-phosphorothioate,
[c] a morpholine ring,
[d] a phosphorodiamidate linkage,
[e] a modification to increase resistance to
RNaseH,
[f] a peptide nucleic acid and
[g] a locked nucleic acid.

AZL Clean Copy of Claims, Paper 8.

2.

To adequately support the claims, the written description “must clearly allow persons of ordinary skill in the art to recognize that the inventor invented what is claimed.” *Ariad Pharm., Inc. v. Eli Lilly & Co.*, 598 F.3d 1336, 1351 (Fed. Cir. 2010) (en banc) (internal quotation marks and brackets omitted). The descriptive text needed varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. *Capon v. Eshhar*, 418 F.3d 1349, 1357 (Fed. Cir. 2005). “[T]he purpose of the written description requirement is to ‘ensure that the scope of the right to exclude, [as set forth in the claims], does not overreach the scope of the inventor's contribution to the field of art as described in the patent specification.’ ” *Ariad* at 1353–54 quoting *Univ. of Rochester v. G.D. Searle & Co.*, 358 F.3d 916, 920 (Fed.Cir.2004)). Thus, the written description plays a vital role in curtailing the permissible claim scope to the actual invention described. *AbbVie Deutschland GmbH & Co., KG v. Janssen Biotech, Inc.*, 759 F.3d 1285, 1299 (Fed. Cir. 2014); *Ariad*, 598 F.3d at 1352.

A specification adequately describes an invention when it “reasonably conveys to those skilled in the art that the inventor had possession of the claimed

1 subject matter as of the filing date.” *Ariad*, 598 F.3d at 1351. In other words the
2 written description requirement ensures that the inventor had possession, as of the
3 filing date of the application relied on, of the specific subject matter claimed.
4 *Chiron Corp. v. Genentech, Inc.*, 363 F.3d 1247, 1255 (Fed. Cir. 2004); *In re*
5 *Wertheim*, 541 F.2d 257, 262 (CCPA 1976). Possession is demonstrated by the
6 disclosure in the specification. *AbbVie*, 759 F.3d at 1299; *Centocor Ortho*
7 *Biotech, Inc. v. Abbott Labs.*, 636 F.3d 1341, 1348 (Fed. Cir. 2011).
8 Determination of possession requires an objective inquiry into the “four corners of
9 the specification from the perspective of a person of ordinary skill in the art.”
10 *Ariad*, 598 F.3d at 1351.

11 For generic claims, there are a “number of factors for evaluating the
12 adequacy of the disclosure, including ‘the existing knowledge in the particular
13 field, the extent and content of the prior art, the maturity of the science or
14 technology, the predictability of the aspect at issue.’” (*AbbVie*, 759 F.3d at 1299);
15 *Capon v. Eshhar*, 418 F.3d 1349, 1359 (Fed. Cir. 2005). Whether a genus is
16 supported vel non depends upon the nature and breadth of the genus. *Hynix*
17 *Semiconductor Inc. v. Rambus Inc.*, 645 F.3d 1336, 1352 (Fed. Cir. 2011).
18 Whether the inventors demonstrated sufficient generality to support the scope of
19 some or all of their claims, must be determined claim by claim. *Capon*, 418 F.3d at
20 1360. “[A] sufficient description of a genus requires the disclosure of either a
21 representative number of species falling within the scope of the genus or structural
22 features common to the members of the genus so that one of skill in the art can
23 ‘visualize or recognize’ the members of the genus.” *Ariad*, 598 F.3d at 1350.

24 The predictability or unpredictability of the involved science is relevant to
25 deciding how much experimental support is required to adequately describe the
26 scope of an invention. (*Capon*, 418 F.3d at 1360). In the “unpredictable” fields of
27 science, it is appropriate to recognize the variability in the science in determining

1 the scope of the coverage to which the inventor is entitled. Such a decision usually
2 focuses on the exemplification in the specification. *Capon*, 418 F.3d at 1358; *Enzo*
3 *Biochem*, 296 F.3d at 1327–28. The appropriate number of exemplified species
4 that one must disclose “necessarily changes with each invention, and it changes
5 with progress in a field.” *Ariad*, 598 F.3d at 1351. “If the difference between
6 members of [a species] is such that [a] person skilled in the art would not readily
7 discern that other [species] of the genus would perform similarly to the disclosed
8 members, i.e., if the art is unpredictable, then disclosure of more species is
9 necessary to adequately show possession of the entire genus.” *Synthes USA, LLC v.*
10 *Spinal Kinetics, Inc.*, 734 F.3d 1332, 1344 (Fed. Cir. 2013) quoting *Bilstad v.*
11 *Wakalopulos*, 386 F.3d 1116, 1125 (Fed.Cir.2004).

12 An *ipsis verbis* disclosure of a claimed genus is not per se sufficient to meet
13 the written description requirement. *Boston Scientific Corp. v. Johnson & Johnson*,
14 647 F.3d 1353, 1364 (Fed. Cir 2011); *Enzo Biochem*, 323 F.3d at 968. “[A]n
15 adequate written description of a claimed genus requires more than a generic
16 statement of an invention's boundaries.” *Ariad*, 598 F.3d at 1349 (citing *Regents of*
17 *the University of California v. Eli Lilly and Co.*, 119 F.3d 1559, 1568 (Fed. Circ.
18 1997)).

19 3.

20 UWA argues that at the time AZL’s applications were filed, identifying
21 AONs that would result in exon skipping was unpredictable. UWA Motion 1,
22 Paper 210, 4:7 – 9:2. UWA argues that there are many factors that influence the
23 binding of an AON to its target that contribute to the unpredictability. UWA
24 Motion 1, Paper 210, 4:8-10. These are said to include the AON sequence length,
25 accessibility to the target, the appropriate portion of the target, nucleobase
26 sequence, nucleotide mismatches between the AON and target, and modifications
27 to the chemical backbone and internucleotide linkages. UWA Motion 1,

1 Paper 210, 4:8-10. As a result, according to UWA, each proposed AON must be
2 empirically tested to verify its ability to cause exon skipping. UWA Motion 1,
3 Paper 210, 4:11-13. Because of this unpredictability, UWA argues, AZL’s single
4 disclosed operative species within the scope of AZL’s claims—the AON
5 designated h53AON1—is insufficient to support the genus of exon 53-skipping
6 AONs encompassed by UZL’s claims. UWA Motion 1, Paper 210, 15:18 – 16. In
7 other words UWA argues that from the disclosure of (1) h53AON1, (2) its ability
8 to cause exon 53 skipping, and (3) the rest of the ’495 application disclosure, one
9 skilled in the art would not conclude that UZL’s inventors had possession of the
10 broad genus of AONs said to be encompassed by AZL’s claims.

11 AZL responds that each of the limitations of its claims is expressly found in
12 its written description. AZL Opposition 1, Paper 392, 6:21 – 7:7. AZL also argues
13 that antisense technology is a mature and predictable field for which AONs are a
14 fundamental tool and that the AON art is sufficiently predictable that the disclosure
15 of the single species h53AON1 in the form a sequence listing provides adequate
16 written descriptive support for the genus claimed. AZL Opposition 1, Paper 392,
17 2:7-10.

18 4.

19 To establish that those skilled in the art considered exon skipping to be
20 unpredictable, UWA relies on the testimony of Dr. Wood. He testifies that exon
21 skipping is unpredictable. Ex. 2081, ¶ 68. In support of his testimony, he
22 identifies a number of publications covering the period 2001 – 2011, a period
23 beginning before and extending after AZL’s September 21, 2005, filing date. We
24 summarize some of these publication below.

25 A 2001 peer-reviewed publication relating to exon 46 skipping titled
26 “Antisense-induced exon skipping restores dystrophin expression in DMD patient

1 derived muscle cells” (Ex. 2012) indicates that it is difficult to predict the AONs
2 that will bind to the target exon:

3 The efficacy of AONs is largely determined by their binding
4 affinity for the target sequence. Due to base composition and
5 pre-mRNA secondary or tertiary structure, *it is difficult to*
6 *predict which AONs are capable of binding the target*
7 *sequence.*

8 Ex. 2012, p. 1548 (emphasis added). The authors tested 12 AONs having
9 overlapping sequences for exon binding and skipping of DMD exon 46 in mouse
10 muscle cells (mAONs 1-12). Ex. 2012, p. 1548. The AONs were complementary
11 to portions of exon 46 differing in specific sequences and sequence length.

12 Ex. 2012, p. 1548, Fig 1B. The mAON’s were said to be 15 or 20 nucleobases in
13 length. Ex. 2012, p. 1550, Table 1. Only five of the twelve, mAONs 4, 6, 8, 9,
14 and 11, were identified as binding to Exon 46. Ex. 2012, p. 1548-50, Figs. 1B, 2A
15 and Table 1. Four of the mAONs —4, 6, 9 and 11—were said to cause skipping of
16 exon 46. Ex. 2012, p. 1548 and Figs. 2C and 2D. mAON 8, which was said to
17 bind to exon 46 and shared an eleven nucleotide sequence with mAON 9 and a
18 seven nucleotide sequence with mAON 11, did not cause exon skipping. Ex. 2012,
19 p. 1548. mAON 10 which apparently shared partial nucleotide sequences with
20 mAON 9 and 11 (Fig. 1B), was not reported as binding with exon 46. Ex. 2012,
21 p. 1548. Additional experiments were reported with respect to human muscle cells
22 using the human AON versions (hAONs) said to correspond to mAONs 4, 6, 8, 9
23 and 11. Ex. 2012, p. 1548-49. The human versions (hAONs) were also 15 or 20
24 nucleotides in length. Ex. 2012, Table 1. With respect to human muscle cells,
25 hAONs 4, 6, 8 and 9 were said to bind, but only 4, 6 and 8 were said to cause
26 skipping. Ex. 2012, p. 1549. hAONs 9 and 11, which share an eleven and seven
27 nucleotide sequences, respectively, with the exon-skipping hAON 8, were not

1 identified as causing exon skipping. Ex. 2012, p. 1549. All the exon-skipping
2 AONs were 15 or 20 nucleobases in length.

3 In a 2002 peer-reviewed article titled “Targeted exon skipping as a potential
4 gene correction therapy for Duchenne muscular dystrophy” (Ex. 2010) identified
5 30 potential AONs for 15 different exons. The authors state that there is no
6 significant correlation between effectiveness and the length or sequence content
7 and that effectiveness of proposed AONs to bind to the desired exon needs to be
8 tested empirically:

9 Of the 30 AONs tested, as many as 20 induced specific exon
10 skipping. *There was no significant correlation between the*
11 *length or sequence content of the AON and its effectiveness (see*
12 *Table 1).* We hypothesize that in most cases the mere
13 accessibility of the targeted RNA region, and thus the capability
14 of the AONs to bind, determines their efficacy. The fact that
15 with the AONs tested so far, we have not been able to induce
16 the skipping of exons 45, 47 and 48 would, in this model, be
17 explained by a less accessible configuration of these exons
18 within the secondary structure of the pre-mRNA. To predict the
19 secondary structure of the targeted pre-mRNA regions, we have
20 used the RNA mfold version 3.1 server. Although this analysis
21 hints at the most favorable local structure which may help in the
22 design of AONs, it is not capable of predicting the overall
23 complex structure of the entire DMD pre-mRNA. *We therefore*
24 *have no insight into the actual position of the targeted sequence*
25 *within the completely folded RNA structure. Its accessibility,*
26 *and thus the effectivity of any designed AON, will therefore still*
27 *have to be tested empirically in the cells, as was done in this*
28 *study.*

29 Ex. 2010, p. S76 (emphasis added). The publication appears to rely on much of the
30 same data presented in AZL’s involved application. Compare Ex. 2010, Tables 1,
31 2 and 3 and Figure 1 with AZL’s involved application, Ex. 1008, Tables 2, 3 and 4
32 and Figure 5, respectively. However, unlike AZL’s application disclosures, the
33 publication does not make any predictions as to additional AONs that include the

1 skip-causing sequences, but also include additional exon-complementary
2 nucleobases. All the AONs reported to have successfully caused exon skipping are
3 reported as 15-24 nucleobases in length. Ex. 2010, Table 1.

4 In another 2002 peer-reviewed publication titled “Improved Antisense
5 Oligonucleotide Induced Exon Skipping in the mdx Mouse Model of Muscular
6 Dystrophy” (Ex. 2017) the authors report making a number of AONs that bound to
7 the region of the dystrophin gene exon 23/intron 23 boundary. Ex. 2017, 646.
8 Three were said to successfully cause skipping. Ex. 2017, p. 631, col. 2. Those
9 AONs were reported to be 17 to 25 nucleobases long.

10 A 2007 peer-reviewed publication titled “Comparative Analysis of
11 Antisense Oligonucleotide Sequences for Targeted Skipping of Exon 51 During
12 Dystrophin Pre-mRNA Splicing in Human Muscle” (Ex. 2013) notes that rules to
13 assist in determining likely candidates for exon skipping in human and mouse
14 muscle cells *in vitro* had yet to be identified although the sequence length and
15 target region are singled out as important:

16 *[S]everal years after the first attempts at dystrophin exon*
17 *skipping with [AONs], there are still no clear rules to guide*
18 *investigators in their design, and in mouse and human muscle*
19 *cells in vitro there is great variability for different targets and*
20 *exons. The consensus sequences at the intron–exon boundaries*
21 *that are involved in splice site selection are only poorly*
22 *conserved, and the [exonic splicing enhancers] that are involved*
23 *in exon definition are themselves of multiple motifs and their*
24 *identification is complex. Until these key elements are better*
25 *understood only length and target region seem to be important*
26 *when designing exon skipping [AONs] for the DMD gene.*

27 Ex. 2013, p. 807 (citation omitted, emphasis added). The successful AONs tested
28 (A20 and B30) were reported as being 20 and 30 nucleobases in length. Ex. 2013,
29 803, Table 2.

1 The 2009 article titled “Guidelines for Antisense Oligonucleotide Design
2 and Insight Into Splice-modulating Mechanisms” (Ex. 2014) notes that
3 notwithstanding the development of various computer programs to assist in
4 identifying AON’s as exon skipping candidates, a trial and error procedure was
5 still necessary. The importance of AON sequence length was also noted:

6 Each antisense mechanism requires stable and efficient binding
7 of the AON to its target sequence. One obvious determinant of
8 AON efficacy is the accessibility of the target Several
9 software programs are available to predict the secondary
10 structure of RNA, of which the m-fold server is the most widely
11 used. This server also provides a so-called SS-count for the
12 target sequence, indicating the propensity of a nucleotide to be
13 single stranded in a number of potential secondary structure
14 predictions. This approach probably reflects the actual *in vivo*
15 situation more closely than focusing only on the most
16 energetically stable structure. In addition, the stability and
17 binding energy of the AON to the target sequence influence
18 AON efficiency. *This depends on e.g., AON length and*
19 *sequence constitution and the free energy of local structures.*
20 To efficiently bind a target sequence, the free energy of the
21 AON-target complex must be higher than that of the target
22 complex and that of the AON. *As AONs are generally only 17–*
23 *25-nucleotides long, they are unlikely to form stable secondary*
24 *structures.* However, most AONs can form AON–AON
25 complexes with other AONs of the same sequence
26 (Supplementary Figure S2). The software program
27 RNAstructure 4.5 has a tool that provides the free energy of
28 AON–AON complexes and AON-target complexes, in addition
29 to the free energy of individual AONs and the target sequence.
30 The aforementioned software programs (as well as others) can
31 be used to facilitate AON design (reviewed in ref. 1).
32 *Nonetheless, none of them is 100% conclusive or predictive and*
33 *in general a trial and error procedure is still involved to*
34 *identify potent AONs.*

35 Ex. 2014, p. 548 (emphasis added, citation and footnote omitted).

1 A 2011 publication titled “Targeted Skipping of Human Dystrophin Exons
2 in Transgenic Mouse Model Systemically for Antisense Drug Development”
3 (Ex. 2015). The authors reported the results of a test using 32 AONs that covered
4 more than two-thirds of human dystrophin exon 50 and its two flanking intron
5 sequences. Ex. 2015, p. 3, paragraph bridging col. 1 and col. 2. The selected
6 AONs had different lengths and were targeted to different portions of exon 50 and
7 its flanking introns. Ex. 2015, Table 1. Thus, all the AONs were antisense to
8 complementary portions of exon 50 and its flanking introns. The results for the
9 25 AONs with 2OMePS backbones are shown in that publication’s Table 1.
10 Ex. 2015, p. 4. Seven of those AONs were identified as causing exon 50 skipping.
11 Review of the Table 1 data shows that the AON length significantly effects exon
12 skipping notwithstanding the inclusion of a common sequence. For example,
13 AONs hE50AO2PS – hE50AO6PS all share the same nineteen base pair sequence.
14 Ex. 2015, Table 1. The 19 and 20 base pair hE50AO2PS and hE50AO3PS were
15 not identified as causing exon skipping. The 22 base pair hE50AO4PS (two
16 additional base pairs) on the 5’ end was said to cause skipping in 4% of cells. The
17 27 base pair hE50AO5PS, with an additional 5 base pairs at the 5’ end over
18 hE50AO4PS, was said to cause skipping in 21%. The 32 base pair hE50AO6PS
19 with 5 more base pairs added to the 5’ end resulted in 3% notwithstanding that the
20 entire 27 base pair sequence of hE50AO5PS is part of the 32 base pair sequence of
21 hE50AO6PS. We find that this data shows that, given the sequence of an AON
22 capable of causing exon skipping, adding and subtracting additional
23 complementary nucleotides significantly effects the capability of the AON to
24 maintain exon skipping. The data also shows that when an AON’s sequence is
25 modified by adding or subtracting a relatively small number of nucleobases, exon
26 skipping is maintained. Compare hE50AO5PS (27 bases) with hE50AO6PS (32

1 bases) and hE50AO4PS (22 bases). Ex. 2015, Table 1. Additionally, the sequence
2 length of all the exon-skipping AONs fall within the range of 17-32 nucleobases.

3 The evidence indicates that at the time AZL filed its application, the
4 identification of AONs that will cause exon skipping was generally thought to be
5 unpredictable. One of the significant factors causing that unpredictability is the
6 effect of the number of nucleobases present in the AON.

7 **5.**

8 AZL also argues that once h53AON1 was identified, one skilled in the art
9 would have investigated extended complementary sequences with the expectation
10 that the longer sequences would bind and cause skipping. AZL Opposition 1,
11 Paper 392, 22:2 – 22:9. AZL directs us to Dr. Sontheimer’s testimony to support
12 its argument. Dr. Sontheimer testifies that “given the proven exon-skipping ability
13 of h53AON1, one of skill in the art would have a high expectation that such AONs
14 of up to 80 nucleotides in length would bind its target and induce exon skipping.”
15 Ex. 1186, ¶ 114, 35:2-5. Dr. Sontheimer does not direct us to any evidence or
16 provide an explanation why one skilled in the art would have had a high
17 expectation. We are not required to credit the unsupported opinions of an expert
18 witness. *Rohm and Haas Co. v. Brotech Corp.*, 127 F.3d 1089, 1092 (Fed. Cir.
19 1997).

20 AZL argues that in considering the permissible scope of genus claims with
21 respect to § 112, the consideration is “the predictability of the aspect at issue.”
22 AZL Opposition 1, Paper 392, 20:2 – 22:9. AZL cites to *Capon*, 418 F.3d at 1359.
23 AZL argues that none of the publications cited by UWA pertain to the
24 predictability of the “aspect at issue.” The aspect at issue is said to be AONs
25 having a sequence capable of binding to and causing skipping of exon 53. AZL
26 Opposition 1, Paper 392, 21: 6-10. According, to AZL the “aspect at issue” is
27 specifically tied to exon 53. AZL Opposition 1, Paper 392, 21:12-16.

1 The basis of our finding on predictability is not inconsistent with *Capon*.
2 We recognize that h53AON1 and SEQ ID NO:29 are central to AZL's claims.
3 However, we are convinced, based upon the evidence reviewed above, that at the
4 time AZL filed its applications, one skilled in the art would not have predicted that
5 exon 53 skipping would be maintained when the h53AON1 sequence length is
6 extended from 18 nucleobases to 50 or 80 complementary nucleobases as specified
7 in AZL's claims. AZL has not directed us to any publications that would provide a
8 basis for that expectation. AZL's witnesses have not identified any publications or
9 other evidence that supports extending the sequence length to the extent specified
10 in the claims.

11 AZL argues that the portions of the publications cited by UWA have been
12 taken out of context. AZL Opposition 1, Paper 392, 21: 3-11. We do not agree.
13 We have evaluated the teachings of the complete publications and find that they
14 support Dr. Wood's opinion that exon skipping is unpredictable, at least with
15 respect to the effect of AON sequence length. As we noted above, AZL's
16 witnesses have not directed us to any publications or other evidence that supports
17 their opinions on predictability.

18 AZL also directs us to test results for AONs of 31, 40 and 50 nucleobases
19 that are said to meet the requirements of AZL's claims and cause exon 53 skipping.
20 AZL Opposition 1, Paper 392, 29:13 – 30:15. The 31 nucleobase AON is UWA's
21 AON having having the nucleobases of its SEQ ID NO:193. AZL Opposition 1,
22 Paper 392, 29:21-23. The tests of the 40 and 50 base AONs were managed and
23 supervised by one of AZL's inventors, Dr. van Deutekom. AZL Opposition 1,
24 Paper 392, 30:1-15.

25 We fail to be persuaded that these tests show that one skilled in the art would
26 have predicted from h53AON1 as of AZL's September 21, 2005, filing date.
27 UWA's tests were not published until after AZL's filing date. UWA's PCT

1 application was not published until January 5, 2006. Thus, those skilled in the art
2 would not have had the benefit of UWA's results. Dr. van Deutekom's tests were
3 carried out in December 2014. Ex. 1125, ¶ 52. Thus, Dr. van Deutekom's
4 unpublished *ex parte* tests are of little probity on how the person of ordinary skill
5 in the art would view predictability of exon skipping as of AZL's September 21,
6 2005, filing date.

7 AZL sites to the "Written Description Training Materials" of
8 March 28, 2008 (Ex. 1068). According to AZL the PTO has determined,
9 apparently as a matter of undisputable fact, that the AON field is predictable. AZL
10 Opposition 1, Paper 392, 4:6-22. AZL quotes from a portion of the Materials that
11 states that because of the high level of skill, those in the field would consider an
12 applicant to be in the possession of the entire breadth of any claimed genus "that
13 could be predicted from the disclosure" based upon a single species AZL
14 Opposition 1, Paper 392, 4:10-13.

15 AZL's reliance on the PTO's training materials is misplaced. First, AZL
16 misapprehends the issue. It is not that AON technology in general is
17 unpredictable. The issue is the unpredictability of AONs that will cause exon
18 skipping. Secondly, the training materials presume predictability of the genus
19 AONs from the species. Ex. 1068, p. 44, penultimate paragraph. A presumption
20 of predictability with respect to exon skipping, especially as to altering the
21 sequence length, is simply contrary to the evidence here.

22 AZL argues once an operative target site is identified, it is straight forward
23 to design longer versions and add additional nucleobases to one or both ends while
24 maintaining complementarity to the pre-mRNA. AZL Opposition 1, Paper 392,
25 4:15-19. AZL relies on the testimony of Dr. Sontheimer, for support. He testifies:

26 A PHOSITA would be able to provide longer sequences simply
27 by starting with the sequence of h53AON1 (SEQ ID NO:29)

1 and using its known complementary sequence within the exon
2 53 pre-mRNA to design and make an oligonucleotide that
3 comprised SEQ ID NO:29 and which contained added bases
4 (either perfectly complementary to its target, or with
5 mismatches) onto either—or both—ends of the SEQ ID
6 NO:29. Thus, according to AZL the design of modified AONs
7 that will cause exon skipping is much more predictable.

8 Ex. 1186, ¶ 95.

9 While we agree that those working in the art could easily design and make
10 additional AONs, and likely would investigate additional AONs, by adding
11 complementary bases to h53AON1, Dr. Sontheimer's testimony does not explain
12 why those working in the art would have predicted or expected that adding, for
13 example, 62 complementary nucleobases to h53AON1 for a total of 80 as stated in
14 AZL's Claim 15, would retain the exon skipping ability of h53AON1. The
15 evidence detailed above establishes that those skilled in the art would have
16 expected that adding a small number of nucleobases to an exon skipping AON
17 would retain a degree of exon skipping, but could not predict the effect of adding a
18 significant number of additional bases. Even as late as 2011, a trial and error
19 approach using AONs having sequences that substantially covered the entire
20 exon 50, was used to determine which AONs would cause exon skipping.

21 Ex. 2015, Table 1. For example, Ex. 2015, Table 1 (AONs hE50AO2PS –
22 hE50AO6PS) shows that adding and subtracting nucleobases while maintaining
23 complementarity with the target sequence significantly changed the ability to cause
24 exon skipping even though all the AONs shared the same 19 nucleobase sequence.
25 Ex. 2015, Table 1.. In other words, even the existence of a common structural
26 feature is not sufficient to predict the presence or maintenance of exon skipping.

27 **6.**

28 Looking at the evidence submitted by the parties relating to predictability of
29 exon skipping, we find that one skilled in the art would have appreciated that there

1 was a substantial degree of unpredictability in changing the sequence length of a
2 known skip-causing AON. We are persuaded that one skilled in the art would not
3 reasonably predict that that AONs of 50 or 80 pre-mRNA exon 53 complementary
4 nucleobases that included the 18 nucleobases of h53AON1 would maintain the
5 exon skipping capability of that AON. Looking to the four corners of AZL's
6 written description, we fail to see an objectively reasonable basis that would permit
7 one skilled in the art to conclude from the single disclosed exon 53-skipping AON,
8 and from the other disclosed AONs said to cause skipping of other exons, that the
9 AZL inventors were in possession of exon 53 skipping AON's having the full
10 range of nucleobases specified in Claims 15, 76, 78-80, 82, 84, 86, 88-90, 97, 98,
11 and 100-103. Because of the unpredictability, AZL's inventors could not have been
12 in possession of the full scope of the subject matter of those claims. We find that
13 AZL's written description, as of its filing date, would not have reasonably
14 conveyed to the person skilled in the art that AZL had possession of the full scope
15 of the AONs having the ranges of nucleobases specified in the claims. We hold
16 that AZL's Claims 15, 76, 78-80, 82, 84, 86, 88-90, 97, 98, and 100-103 are not
17 supported by an adequate written description and are unpatentable under 35 U.S.C.
18 § 112(a).

19 **7.**

20 We come to a different conclusion with respect to AZL's dependent
21 Claim 77. That claim is expressly limited to an AON having the 18 nucleobase
22 sequence represented by SEQ ID NO: 29. While the claim is limited to the
23 specific 18 nucleobase sequence, the claim permits a variation in the modified
24 AON backbone. It lists 5 categories of backbones in addition to 2OMePS. AZL's
25 Clean Copy of Claims, Paper 8, Claims 15 and 77, 1:3-8 and 1:17-17. UWA argues
26 that the variation in the backbone chemistry contributes to the unpredictability of
27 the AONs that will cause exon skipping. UWA Motion 1, Paper 210, 1: 14-16.

1 UWA directs us to Exhibits 2020 and 2013 and ¶¶ 75 and 77 of Dr. Wood's
2 testimony to establish, *inter alia*, that the choice of backbone effects the degree to
3 which the AONs will cause exon skipping. UWA Motion 1, Paper 210, 5:17 – 7:3.

4 With respect AZL Claim 77, UWA has not established that one skilled in the
5 art would conclude that AZL's inventors did not have possession of the full scope
6 of the subject matter of Claim 77. The evidence does not establish that the effect
7 of substituting some or all of the 2OMePS backbone of h53AON1 with the other
8 backbone materials recited in the claims renders the effect of the substitution on
9 exon skipping unpredictable. As noted by Dr. Wood:

10 51. In an effort to cope with the many requirements for exon
11 skipping, scientists have explored many different AON
12 chemistries, including AONs with modifications to the
13 nucleobase, the backbone, the internucleotide linkages, and
14 combinations of each.

15
16 52. AON chemistries can vary significantly from naturally
17 occurring nucleotides. However, they preserve the ability to
18 form Watson-Crick base pairs with pre-mRNA through the
19 maintenance of nucleobases (sometimes modified) in the
20 correct spatial conformation.

21 Ex, 2081, ¶¶ 51-52. Dr. Wood then discusses a number of known substitute
22 backbones, explaining the effect of each on the properties of the AON. Ex. 2081,
23 ¶¶ 52-60. AZL's Dr. Sontheimer similarly testifies that the effect of the backbones
24 on the AONs was known at the time the AZL's '495 application was filed.

25 Ex. 1186, ¶¶ 119-124. While the record shows modifying the 2OMePS backbone
26 of h53AON1 would likely change the efficiency, i.e., the degree of skipping, we
27 are not convinced that the expected change in efficiency on h53AON1 is sufficient
28 to hold that the changes are so unpredictable that AZL's inventors were not in
29 possession of the full scope of the subject matter of Claim 77. As noted by
30 Dr. Wood, the changes in the chemistry "preserve[s] the ability to form Watson-

1 Crick base pairs with pre-mRNA through the maintenance of nucleobases
2 (sometimes modified) in the correct spatial conformation. “ Ex. 2081, ¶ 20:52.
3 His testimony is consistent with Ex. 2020 that teaches that differences in skipping
4 efficiency between AONs with different backbones appear to be sequence and not
5 chemistry (i.e., backbone) dependent, although certain backbones may be less
6 sequence dependent than others:

7 [D]ifferences in efficiency between PMO and 20MePS appear
8 to be sequence and not chemistry dependent. Finally, the results
9 indicate that PMOs may be less sequence specific than
10 20MePS.

11 Ex. 2020, p. 257 (Conclusions).

12 UWA has failed to meet its burden to show that the subject matter of the full
13 scope of Claim 77 was not in the possession of AZL’s inventors and lack written
14 descriptive support.

15 **B.**

16 UWA argues that AZL’s application fails to enable one skilled to make and
17 use the full scope of the claimed invention. UWA Motion 1, Paper 210, 24:12-13.
18 UWA bases its argument on the scope of UWA’s claims, the unpredictability of
19 exon skipping, the amount and nature of experimentation necessary and the lack of
20 clinical data to establish therapeutically useful AONs. UWA Motion 1, Paper 210,
21 25:21 – 30:21. We held above that there is a significant degree of unpredictability
22 with respect to exon skipping, at least with respect to AON sequence length.

23 UWA says that undue experimentation is required due primarily to
24 unpredictability and the very large number and variety of potential AONs covered
25 by the claims that would need to be tested. UWA Motion 1, Paper 210, 28:1 –
26 30:3. Making and testing even a small number of possible AONs, says UWA,
27 requires a massive investment in time and effort. UWA Motion 1, Paper 210,
28 28:19 – 29:4.

1
2 **1.**

3 UWA again relies on Dr. Wood’s testimony. He testifies that due to the
4 unpredictability of exon skipping, and due to the potential mismatches between the
5 AON and pre-mRNA, different possible backbones, and sequence length of the
6 AONs, a very large number of AONs is potentially covered by the claims. He
7 presents a calculation of the number of possible AONs covered by the claims.
8 Ex. 2081, ¶ 192. He further testifies that a large number of potentially covered
9 AONs would need to be synthesized and tested. Ex. 2081, ¶ 422. As a result a
10 massive amount time and effort would be necessary to perform this testing.
11 Ex. 2081, ¶ 423.

12 AZL argues that the person of ordinary skill in the art would have been able
13 to readily make the AONs “recited in the ‘495 application.” AZL Opposition 1,
14 Paper 392, 12:20-21. It further argues that in light of the high level of skill in the
15 art, the public availability of the sequence of exon 53, the examples in AZL’s
16 specification of the effectiveness of the 2OMePS AONs, a person of ordinary skill
17 “would have been capable of using the [AONs] of the ’495 claims to induce exon
18 [53 skipping.]” AZL Opposition 1, Paper 392, 14:3-8. With respect to the amount
19 of experimentation, AZL argues that a reasonable amount of routine experiment
20 does not violate the enablement requirement and having disclosed the sequence of
21 h53AON1, only routine testing was necessary to see if exon skipping was induced.
22 AZL Opposition 1, Paper 392, 14: 23 – 16:13.

23 While we view UWA’s computation of the number AONs covered by the
24 claims (Ex. 2081. ¶¶ 191-195) to be overly speculative, AZL does not appear to
25 challenge that its claims encompass an extremely large number of possible AONs.
26 We are persuaded that a very large number of AONs would need to be synthesized
27 and tested for exon skipping activity and would amount to undue experimentation.

1 Undue experimentation is a matter of degree. *Chiron Corp. v. Genentech,*
2 *Inc.*, 363 F.3d 1247, 1253 (Fed. Cir. 2004). While necessary testing may be
3 routine, even routine testing may be undue if it involves synthesizing and testing a
4 very large number of candidates. *Wyeth and Cordis Corp. v. Abbott Laboratories,*
5 720 F.3d 1380, 1385-86 (Fed. Cir. 2013).

6 While routine experimentation, even if difficult and time consuming, does
7 not mandate a conclusion that the amount of experimentation is undue (*Falkner v.*
8 *Inglis*, 448 F3d 1357, 1365 (Fed. Cir. 2006)), we view this case to be very similar
9 to *Wyeth and Cordis*. There the claims were directed to a treatment for preventing
10 restenosis using a class of compounds called rapamycin. Rampamycins include a
11 specific macrocyclic triene ring. The patents involved disclosed a single
12 rapamycin compound which included a specific substituent at one location of the
13 ring. The evidence showed that because of the large number of possible
14 modifications at the substituent site on the ring there were a very large number of
15 potential candidate rapamycins. Each potential rapamycin would need to be tested
16 to see if it prevented restenosis. The court held that the amount of testing under
17 these facts indicated a degree of experimentation that was undue. Similarly, the
18 record here, shows that exon skipping has a significant degree of unpredictability
19 and that it is uncontested that there are a very large number of possible AONs
20 within the scope of AZL's claims and a very large number of AONs would need to
21 be manufactured and tested to determine whether exon skipping capability was
22 present. In our view because of the unpredictability of exon skipping, and the
23 large number of potential candidate AONs within the scope of the claims, an undue
24 amount of experimentation would be required to practice the full scope of AZL
25 Claims 15, 76. 78-80, 82, 84, 86, 88-90, 97, 98, and 100-103. Those claims are
26 unpatentable under 35 U.S.C. § 112(a).

1 We again reach a different conclusion with respect to Claim 77. As we
2 noted above, this claim is much more limited in its scope. It is limited to the
3 specific 18 nucleobase sequence of SEQ ID NO: 29. While the specific backbone
4 may be varied, the evidence discussed above with respect to the written description
5 shows that those working in the art were aware of the effects of modifying the
6 backbone.

7 UWA has not established that an undue amount of experimentation would
8 have been necessary to make and test AONs having the 18 nucleobase sequence of
9 SEQ ID NO: 29 with the different backbones recited Claim 77. UWA has failed to
10 meet its burden of establishing that AZL's Claim 77 is not enabled for its full
11 scope.

12 **C.**

13 UWA motion 1 is granted with respect to Claims 15, 76, 78-80, 82, 84, 86,
14 88-90, 97, 98, and 100-103, but denied with respect to Claim 77.

15 **V.**

16 *UWA Motion 2 – Unpatentability under 35 U.S.C. § 112(b)*

17 **A.**

18 UWA “requests entry of judgment that [AZL’s] involved Claims 15, 76-80,
19 82, 84, 86, 88-90, 97, 98, 100-103 in Application No. 11/233,495 . . . are indefinite
20 and therefore unpatentable under 35 U.S.C. § 112(b).” UWA Motion 2, Paper 211,
21 1: 2-5. UWA argues that the claims do not inform a person of ordinary skill in the
22 art of the level of skipping, the conditions for testing skipping, and how to measure
23 or evaluate exon skipping. According to UWA, different techniques can be used to
24 measure exon skipping. An AON might meet the claim requirements when
25 considered by one technique while in another it would not, thus resulting in
26 indefinite claims:

1 [A]pplying a broad interpretation of the claims where any
2 means of measuring exon skipping is permitted, some AONs
3 *may* fall within the scope of the “comprising” claims, but when
4 exon skipping is measured in another way, they *may* not. This is
5 the essence of ambiguity.

6 UWA Motion 2, Paper 211, 17: 7-9 (emphasis added). As a result, UWA says,
7 AZL’s claims are indefinite under *Honeywell Int’l, Inc. v. ITC*, 341 F.3d 1332
8 (Fed. Cir. 2003). More specifically, UWA argues that the claims and specification
9 do not provide guidance on how to determine if exon skipping was induced or how
10 exon skipping is to be measured or detected. UWA Motion 2, Paper 211, 19:4 –
11 20:18

12 **B.**

13 The Supreme Court has explained the definiteness requirements of § 112 in
14 *Nautilus, Inc. v. Biosig Instruments, Inc.*, ___ U.S. ___, 134 S. Ct. 2120, 2129
15 (2014).

16 [W]e read § 112, ¶ 2 to require that a patent's claims, viewed in
17 light of the specification and prosecution history, inform those
18 skilled in the art about the scope of the invention with
19 reasonable certainty. The definiteness requirement, so
20 understood, mandates clarity, while recognizing that absolute
21 precision is unattainable. The standard we adopt accords with
22 opinions of this Court stating that “the certainty which the law
23 requires in patents is not greater than is reasonable, having
24 regard to their subject-matter.” *Minerals Separation, Ltd. v.*
25 *Hyde*, 242 U.S. 261, 270, 37 S.Ct. 82, 61 L.Ed. 286 (1916).

26 134 S. Ct. at 2129. UWA, who bears the burden of proof (37 CFR
27 § 41.208(b)), must therefore establish that AZL’s claims do not inform those
28 skilled in the art of the scope of the claims with “reasonable certainty.”

29 **C.**

30 With respect to Claims 15, 76, 80, 82, 84, 86, 88-90, 98, 101, and 102 UWA
31 directs us to Dr. Wood’s testimony. He testifies that AZL’s claims encompass a

1 very large number of possible AONs and that exon skipping is unpredictable.
2 Ex, 2081, ¶¶ 455-457. He also testifies that under different testing conditions, an
3 AON “might” be identified as causing exon skipping under one set of condition but
4 it “might” not be identified under different conditions:

5 When exon skipping is measured under one set of conditions an
6 AON meeting the structural features of the claims might be
7 found to meet the structural requirement of inducing skipping,
8 but when measured under another set of conditions it may not.

9 Ex. 2081, ¶ 458.

10 **D.**

11 We are not convinced by UWA’s arguments and evidence.

12 **1.**

13 We do not think that *Honeywell* is applicable under the facts here. In
14 *Honeywell*, the claims required a specific numerical range for “melting point
15 elevation” (MPE). The evidence showed that depending on the specific way MPE
16 was tested, the same sample would either be within or outside the specified range.
17 *Honeywell*, 341 F.3d at 1336. The court held that the Honeywell’s claims, which
18 did not identify the method of testing MPE, were indefinite with respect to the
19 method of how the product was to be tested. *Honeywell*, 341 F.3d at 1340. Unlike
20 the claims involved in *Honeywell*, AZL’s claims do not require any specific degree
21 of exon skipping. They only require that the AONs cause, or are capable of
22 causing, some amount of exon skipping. Thus, any degree of skipping will bring
23 the AON within the scope of the skipping requirement of the claims. This fact
24 alone distinguishes AZL’s claims from those involved in *Honeywell*. Additionally,
25 unlike *Honeywell*, UWA has not directed us to evidence establishing that different
26 tests would in fact lead to different results for the same AON.

2.

Additionally, while Dr. Wood testifies to the possibility of variability in results for different tests, we have not been directed to evidence sufficient to establish that one having ordinary skill in the art would not be able to determine whether an AON caused exon 53 skipping notwithstanding the variability in the results of different tests. The level of skill in the art is very high. As stated by Dr. Wood:

A person of ordinary skill in the art would have a Ph.D. degree in cell biology, genetics, molecular biology, or an equivalent, and several years of experience with AONs for inducing exon skipping, including familiarity with *in vitro* and *in vivo* methods for testing the safety and efficacy of such AONs. Further, a person of ordinary skill in this art would have at least some knowledge of, and experience with, chemical modifications that may be incorporated into AONs, such as modifications to the backbone and/or nucleobases of the AONs, and the impact of those modifications on the utility of the AONs. The person of ordinary skill in the art would also have at least some understanding of the use of AONs for inducing exon skipping in the context of medical conditions, such as DMD, that may be treated by administering such AONs.

Ex. 2081, ¶ 179. Those working in this field are aware of an array of different tests to determine exon skipping. Ex. 1008, 24:4 – 26:6. The evidence does not establish that those highly skilled scientists are not aware of the limitations and the appropriate use of each of these routinely used tests and would not be able to accurately assess whether exon skipping was actually present or not.

Additionally, we note that, at least where standardized protocols were used, the different tests reliably show comparable results:

Results from the different laboratories were highly concordant with minimal inter- and intralaboratory variability, particularly with quantitative immunohistochemistry. There was a good level of agreement between data generated by

1 immunohistochemistry and Western blotting, although
2 immunohistochemistry was more sensitive. Furthermore, mean
3 dystrophin levels determined by alternative quantitative
4 immunohistochemistry methods were highly comparable.

5 Ex. 2028, p. 1. Thus, notwithstanding variability in test results, it appears one
6 skilled in the art would still reliably determine whether or not an AON induced
7 exon skipping.

8 The evidence here fails to establish that the variability in the results of
9 different tests would result in those skilled in the art being unable to reasonably
10 determine whether and AON meet the claim requirement of “inducing exon
11 skipping.” Claims 15, 76, 80, 82, 84, 86, 88-90, 98, 101, and 102 have not been
12 shown to be indefinite.

13 **E.**

14 UWA also argues that the phrase “capable of binding to an exon-internal
15 sequence of exon 53” used in Claims 78, 79, 82, 84, 86, 88-90, 97, 98, and 100-
16 103 renders those claims indefinite. UWA Motion 2, Paper 211, 21:15 – 25:20.
17 Relying on the same arguments it made with respect to detecting exon skipping,
18 UWA says the “capable of binding” is indefinite because the claims “fail to recite
19 the conditions for testing, detecting and evaluating exon skipping.” UWA
20 Motion 2, Paper 211, 22:5-8. We held above that UWA’s argument with respect to
21 “inducing exon skipping” was unpersuasive. In view of the high level of skill in
22 the art, we are not convinced that one skilled in the art could not reasonably
23 evaluate whether an AON is capable of binding to exon 53 for the reasons stated
24 above with respect to detecting exon skipping.

25 Additionally, we note that, “capable of binding” and its equivalent “capable
26 of hybridizing” are ubiquitously used in the involved technology. Indeed, both
27 parties use the phrases in their respective written descriptions to describe the
28 invention. For example, UWA says: “The present invention describes antisense

1 molecules capable of binding to specified dystrophin pre-mRNA targets and re-
2 directing processing of that gene.” Ex. 2046, 23:27-29. AZL similarly teaches:
3 “An oligonucleotide capable of hybridizing to pre-mRNA at a location of an exon
4 that is normally included in the mature mRNA can direct the exclusion of the thus
5 targeted exon or a part thereof.” Ex. 1008, 1:27-29. The evidence to which we
6 have been directed is insufficient to establish that those working in the art would
7 not understand these often used phrases as they are used in the context of AZL’s
8 specification.

9 **F.**

10 UWA also argues that because “exon-internal sequence” is undefined,
11 Claims 78, 79, 82, 84, 86, 88-90, 97, 98, and 100-103 which include that phrase are
12 indefinite. UWA Motion 2, Paper 211, 22:9 – 23:18. UWA’s Dr. Wood testifies
13 that the AZL’s applications do not provide any guidance on the meaning of the
14 phrase. Ex. 2081, ¶ 209. AZL says “internal means ‘within’ the exon, which also
15 means located between the ends of exon 53” Paper 396, 9:4-6. AZL’s,
16 Dr. Erik Sontheimer, testifies that an exon-internal sequence “does not include an
17 exon/intron boundary or an intronic sequence” Ex. 1067, ¶ 17.

18 While AZL’s specification does not appear to define the phrase, UWA does
19 not explain why the phrase needs definition. The phrase must be considered in the
20 context of the claim in which it appears (*IGT v. Bally Gaming Int’l, Inc.*, 659 F.3d
21 1109, 1117 (Fed. Cir. 2011)) and of the specification of which it is apart (*Phillips*
22 *v. AWH Corp.*, 415 F.3d 1303, 1313 (Fed.Cir.2005) (*en banc*)). The literal meaning
23 of the words, as used in the phrase “exon-internal sequence of exon 53” specifies a
24 sequence that is inside of, or in the interior portion of, exon 53. AZL’s
25 construction is consistent with the literal meaning of the phrase in the context of
26 the claim. UWA has not directed us to sufficient evidence that those working in
27 the art would understand the phrase in a manner different than its literal meaning

1 of the words when read in the context of the claim and the rest of the specification.
2 Nor has UWA provided a basis as to why the phrase should be given a connotation
3 different than the literal meaning. We construe “exon internal sequence” to mean a
4 sequence that is inside the exon but excludes that two bounding nucleobases at the
5 3’ and 5’ ends of the 212 nucleobase exon 53. In other words, the exon internal
6 sequence is 210 nucleobase sequence of exon 53 but excluding its ending 5’ and 3’
7 nucleobases.

8 UWA has failed to show that the phrase “exon-internal sequence” is not
9 clear and does not inform one skilled in the art of the scope of the claims with
10 reasonable certainty.

11 **G.**

12 UWA also argues that Claims 78, 79, 82, 84, 86, 88-90, 97, 98, 100 and 103
13 are indefinite because these claims require that the AONs must be capable of
14 binding to an exon-internal sequence of exon 53 to which h53AON1 is also
15 capable of binding. The claims are said to be indefinite because

16 no conditions are specified in the claims, the application, or the
17 file history, for determining whether h53AON1 is capable of
18 binding to the “exon-internal sequence” sequence” that is bound
19 by the claimed AON. (Exh. 2081 at ¶ 449.) Also, there is no
20 disclosure in the AZL applications concerning how “binding”
21 of the AON to the “exon-internal sequence” of the pre-mRNA
22 is assayed or measured. (Exh. 2081 at ¶ 446.) The AZL
23 applications do not describe how to reach a conclusion that the
24 AON has bound, other than by reference to the separate and
25 distinct functional requirement recited in claims that exon
26 skipping is induced. (Exh. 2081 at ¶ 449.) Nor does the
27 intrinsic evidence inform a person of ordinary skill in the art
28 whether the same conditions for binding of the claimed AON
29 and h53AON1 are required, or if different conditions may be
30 used. (Exh. 2081 at ¶ 449.) This might be expected as
31 “binding” is never directly measured in the AZL applications.

1 UWA Motion 2, Paper 211, 23:22 – 24:9. UWA again relies on Dr. Wood’s
2 testimony for support. He testifies that binding of nucleic acids is “exquisitely”
3 sensitive to the conditions employed and there is no way to know if binding occurs
4 because the conditions are not specified. Ex. 2081, ¶ 247.

5 We do not credit Dr. Wood’s testimony on this point. His referenced
6 testimony does not direct us to evidence in support. We are not required to credit
7 unsupported expert testimony. *Rohm and Haas*, 127 F.3d 1089, 1092 (Fed. Circ.
8 1997). *See also, Phillips v. AWH Corp.*, 415 F.3d at 1318 (“[C]onclusory,
9 unsupported assertions by experts as to the definition of a claim term are not useful
10”) and *Aristocrat Techs. Austl. PTY Ltd. v. Int’l Game Tech.*, 709 F.3d 1348,
11 1360-61 (Fed. Cir. 2013) (an explanation of only “how” a person of ordinary skill
12 would understand a claim term without an explanation of “why” is “not useful”
13 and should be discounted). Dr. Wood does not direct us to sufficient evidence
14 supporting his testimony that the conditions of binding in either the claims or the
15 specification are necessary. As we noted above, the level of ordinary skill in the art
16 is very high. Based on the evidence to which we have been directed, we fail to see
17 that those working in the art would not use routine testing and conditions to
18 perform the necessary comparison between the AON and h53AON1. The fact that
19 some experimentation may be necessary to determine whether an AON falls within
20 the scope of the claims does not render the claims indefinite. *Exxon Research and*
21 *Engineering Co. v. U.S.*, 265 F.3d 1371, 1379 (Fed. Cir. 2001); *W.L. Gore &*
22 *Assocs., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1557 (Fed.Cir.1983).

23 **G.**

24 UWA has failed to satisfy its burden of establishing that AZL’s Claims 15,
25 76-80, 82, 84, 86, 88-90, 97, 98, 100-103 fail to inform persons skilled in the art of
26 the scope of the claims with reasonable certainty. Accordingly, those claims have
27 not been shown to be indefinite under 35 U.S.C. 112(a).

1 **VI.**

2 *UWA motion for the declaration of an additional interference*

3 UWA moves for the declaration of an additional interference between its
4 currently involved patent, 8,455,636, and another AZL application, 14/248,279.
5 The '279 application is said to be a continuation of AZL's involved '459
6 application. Because UWA has not established that it has a basis upon which it
7 could prevail on priority, we deny the motion.

8 **A.**

9 The Claims of the '279 application are directed to a method of inducing
10 exon 53 skipping, an expression vector encoding a transcript comprising an AON
11 capable of binding to exon 53 and a gene delivery vehicle comprising that vector.
12 Like AZL's involved claims in this interference, the subject matter of the '279
13 application claims centers on SEQ. ID. NO: 29 and the AON designated
14 h53AON1. Ex. 2053, pp. 2-4.

15 **B.**

16 A party in an interference may suggest the declaration of an additional
17 interference:

18 A party may suggest . . . the declaration of an additional
19 interference. The suggestion should make the showings
20 required under § 41.202(a) of this part.

21 37 CFR § 41.203(d). Section 41.202(a) provides in relevant part:

22 An applicant, including a reissue applicant, may suggest an
23 interference with another application or a patent. The suggestion
24 must: . . . (4) Explain in detail why the applicant *will prevail on*
25 *priority*

26 37 C.F.R. § 41.202(a) (emphasis added). Paragraph (e) of § 41.202, further
27 requires:

(e) Sufficiency of showing. (1) A showing of priority under this section is not sufficient unless it would, if unrebutted, support a determination of priority in favor of the party making the showing.

37 C.F.R. § 41.202(e). Priority may be established by showing an earlier reduction to practice, or an earlier conception coupled with diligence to a later reduction to practice. 35 U.S.C. § 102(g) (2011). The reduction to practice may be constructive or actual. *Cooper v. Goldfarb*, 154 F.3d 1321, 1327 (Fed. Cir. 1998).

The mechanism for suggesting an additional interference is a miscellaneous motion. Standing Order, Paper 2, ¶ 203.1. As noted in the Standing Order: “The motion must comply with the requirements of [37 C.F.R. § 41.]202(a) *even for a patent.*” *Id* (emphasis added). The party filing a motion has the burden of proof. 37 CFR §§ 41.121 (b) and 41.208(b).

C.

UWA argues that “[s]ince all of the AZL claims are unpatentable for a lack of written description and enablement, priority is not an issue.” UWA Motion 3, Paper 212, 7:4-5. UWA also argues that

the AZL ’279 application, the AZL ’495 application and the AZL PCT application from which the AZL ’279 application claims benefit, do not adequately describe or enable the claims in the AZL ’279 application. As such, AZL is not entitled to a filing date earlier than its April 8, 2014 filing date. (Ex. 2081, ¶¶279-433). As such, UWA will prevail in the interference.

UWA Motion 3, Paper 212, 7:10-14. Raising essentially the same arguments that it raised with respect to its Motion 1 (Paper 210), UWA argues that the claims of AZL’s ’279 application are unpatentable under 35 U.S.C. § 112 for failing to meet the written description and enablement *for the full scope* of the claimed subject matter. UWA Motion 3, Paper 212, 12:8 – 15:5. UWA premises its argument on the assertion that AZL’s ’279 application and its parent applications “disclose just

1 a single species of AON allegedly capable of inducing *in vitro* skipping of
2 exon 53” UWA Motion 3, Paper 212, 13:6-8 and 14:9-12. UWA does not
3 assert that that the “single species” is not adequately disclosed in those applications
4 or does not meet the limitations of Proposed Count 2.

5 **D.**

6 UWA’s Motion 3, does not attempt to show an earlier actual reduction to
7 practice or an earlier conception coupled with diligence. Rather, it argues that each
8 of its earlier applications is a constructive reduction to practice of the subject
9 matter of Proposed Count 2 entitling it to an effective filing date of June 28, 2005.
10 UWA Motion 3, Paper 212, 6:16 - 7: 9. On the other hand, argues UWA, AZL is
11 not entitled to a date earlier than the April 8, 2014, filing date of the ’279
12 application. UWA Motion 3, Paper 212, 7: 10-14. The reason, according to
13 UWA, is that

14 the AZL ’279 application, the AZL ’495 application and the
15 AZL PCT application from which the AZL ’279 application
16 claims benefit, do not provide written description or enablement
17 for the claims in the AZL ’279 application.

18 AZL’s claims are said not to be supported for the “full scope” of the subject matter
19 claimed. UWA Motion 3, Paper 212, 12:8 – 15:5.

20 Assuming that UWA is correct about the unpatentability of AZL’s ’279
21 claims under 35 U.S.C. § 112(a) due to the lack of support for the full scope in
22 ’279 application and in UWA’s parent applications, AZL has failed to establish
23 that it will prevail on priority. 37 C.F.R. §§ 41.203(d), 42.202(a), 41.202(e);
24 SO ¶ 03.1.

25 UWA notes that AZL’s ’279 application claims benefit of AZL’s
26 ’495 application (involved herein) and PCT application PCT/NL2003/000214 filed
27 March 21, 2003. Those applications are said to “disclose just a single species of
28 AON allegedly capable of inducing *in vitro* skipping of exon 53” UWA

1 Motion 3, Paper 212, 13:6-8 and 14:9-12. UWA does not allege that the
2 disclosures of that single species (h53AON1) in the '279 application and AZL's
3 earlier applications, in the context of the specifications of those applications, does
4 not provide a written description and enabling disclosure of an embodiment
5 meeting the limitations of UWA's Proposed Count 2.¹ Thus, UWA's has not
6 shown the '279 application and each of its parent applications, is not a constructive
7 reduction to practice of an embodiment meeting the limitations of the proposed
8 count.² For the purpose of priority in an interference, where a "parent application
9 is relied upon as a prior constructive reduction to practice[,] . . . the § 112, first
10 paragraph requirements need only be met for an *embodiment* within the count."
11 *Hunt v. Treppschuh*, 523 F.2d 1386, 1389 (CCPA 1975). *See also, Falkner v.*
12 *Inglis*, 448 F.3d 1357, 1362 (Fed. Cir. 2006). UWA's earliest claimed benefit
13 application is AU 2004 903474, filed June 28, 2004, a date subsequent to AZL's
14 earliest possible constructive reduction to practice (PCT application
15 PCT/NL2003/000214 filed March 21, 2003). UWA has not provided argument nor
16 directed us to evidence sufficient to establish a reasonable basis upon which it will
17 prevail on priority.

18 A junior party seeking an interference may have a basis to assert that an
19 opponent's claimed subject matter is not patentable. However, without a colorable
20 basis for a junior party to prevail on priority, there is simply an inadequate
21 foundation to invoke the Board's interference jurisdiction to address patentability.
22 Allegations of unpatentability, on their own, do not provide an adequate
23 justification for the Office to exercise discretion to allow an interference to proceed

¹ Proposed Count 2 is set out in Appendix A to UWA's motion (Paper 212).

² When this interference was declared AZL was accorded the benefit of the filing dates of its earlier application back to its PCT application PCT/NL2003/000214 filed March 21, 2003.

solely to address patentability issues. *See* 35 U.S.C. § 135(b) (2010) (“The Board of Patent Appeals and Interferences shall determine questions of priority of the inventions and may determine questions of patentability.”)

E.

UWA’s Motion 3 requesting an additional interference between Patent 8,455,636 and AZL Application 14/248,279 is denied.

VII.

AZL’s motion for unpatentability over prior art

AZL moves for a judgment that UWA’s involved claims are unpatentable on the following grounds:

1. Claims 1–12, 14–16, 19–29, 31–33, 37 and 38 under 35 U.S.C. § 102(e) or § 103(a) over International Published Application WO2004/0834321 (“VO”), relying additionally on two Koenig et al publications (collectively Koenig) as evidence supporting anticipation or to establish obviousness;
2. Claims 16–18 and 33–35 under § 103(a) as unpatentable over the combined teachings of VO, Koenig and International Published Application WO 2001/72765 (Bennett).
3. Claims 13 and 30 under § 103(a) over the combined teachings of VO, K1, K2 and WO 1994/26887 (Kole);
4. Claims 36 and 39–43 under § 103(a) over the combined teachings of VO, Koenig and WO 00/15780 (Latchman).

A.

UWA Claims 1, 4, 19 and 20

UWA’s involved Claim 1 is directed to an AON of 20-50 nucleobases that hybridizes to a region of exon 53 of the human dystrophin gene causing exon 53 to be skipped. The AON must include at least 20 consecutive nucleobases of UWA’s

1 SEQ ID NO: 193. Additionally, the uracil bases may be substituted with thymine.

2 We reproduce UWA's Claim 1 below with paragraphing added:

3 1. An isolated antisense oligonucleotide
4 of 20 to 50 nucleotides in length comprising
5 at least 20 consecutive nucleotides of SEQ ID NO:193,
6 wherein the oligonucleotide specifically hybridizes to an exon
7 53 target region of the human dystrophin gene
8 inducing exon 53 skipping, and
9 wherein the uracil bases are optionally thymine bases

10 UWA Clean Copy of Claims, Paper 12, App A-1, Claim 1. SEQ ID NO:193 also
11 has the following 31 nucleobase sequence:

12 CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G

13 Ex. 1003, Table 1A, col. 17; Table 3A, 56:52-54. That sequence is also designated
14 by its annealing site H53A(+39 + 69). *Id.* UWA's other independent claim,
15 Claim 19, is essentially identical to Claim 1 except that it identifies the sequence
16 by the annealing site H53A(+39 + 69) rather than referring to SEQ ID NO:193.

17 UWA Clean Copy of Claims, Paper 12, App A-3, Claim 19. Claims 2 and 20 limit
18 the AONs to 20 to 31 nucleotides. UWA Clean Copy of Claims, Paper 12, App A-
19 3, Claims 2 and 20.

20 **1.**

21 AZL relies on International Published Application WO2004/0834321
22 ("VO"). It is of record as Ex. 1007. Its status as prior art to UWA's claimed
23 subject matter is not challenged. VO is the published version of the specification
24 of AZL's International Application PCT/NL03/000214. Ex. 1007, p. 1. AZL's
25 involved application claims to be a continuation of that international application.

26 AZL principally relies on VO's disclosure of h53AON1. As we discussed
27 above, that AON is described as having an eighteen nucleobase sequence and said
28 to cause exon skipping of exon 53. Ex. 1007, Table 2, 48:33. UWA's SEQ ID

1 NO: 193 is a 31 nucleobase sequence that includes the 18 bases of h53AON1.

2 Ex. 1003, Table 3A, 56:52-54. We compare the sequences below with the
3 identical portions shown in bold:

4 SEQ ID 193 (31mer): cauuc aacug **uugcc uccgg uucug** aaggx g
5 VO (h53AON1 18mer): **cug uugcc uccgg uucug**

6 One difference between h53AON1 as described in VO differs from the AON of
7 UWA's Claim 1 and 19 is the number of nucleobases. h53AON1 has 18. UWA's
8 Claims 1 and 19 require at least 20 consecutive nucleobases of SEQ ID NO:193 or
9 of annealing site H53A(+39+69). VO also teaches that the AONs are "preferably
10 complementary to a consecutive part of between 16 and 50 nucleotides of said
11 exon RNA." Ex. 1007, 9:28-30. It is uncontested that the Koenig references
12 (Exs 1010 and 1011) teach the complete sequence of exon 53 was known to those
13 working in the art. Given h53AON1, AZL argues, it would have been obvious to
14 make AONs having different lengths that included the sequence of h53AON1 but
15 still complementary to exon 53. The person having ordinary skill in the art would
16 have been motivated to investigate AONs of different lengths in order to determine
17 the optimum length for exon skipping. Ex. 1012, ¶ 56. Thus, according to AZL,
18 UWA's Claims 1, 4, 19 and 20 encompass subject matter that would have been
19 obvious and are unpatentable under 35 U.S.C. § 103(a)

20 UWA argues that there is no rationale provided in the combined teachings of
21 the references to modify h53AON1 to produce an AON within the scope of
22 UWA's claims. UWA Opposition 1, Paper 393, 22:19-22. UWA also argues that
23 because of the unpredictability of exon skipping there would be no reasonable
24 expectation of success in making any modifications. *Id.* at 22:21-23.

25 2.

26 VO specifically teaches that AONs are "preferably complementary to a
27 consecutive part of between 16 and 50 nucleotides of said exon RNA." Ex. 1007,

1 9:28-30. In our view, that language at least suggests to one having ordinary skill in
2 the art to modifying an AON that has been disclosed to cause exon skipping by
3 changing its sequence length by adding additional exon 53 pre-mRNA
4 nucleobases.

5 We find no merit in UWA's argument that VO's method of generating
6 AONs does not include taking a known exon-skipping AON and adding
7 nucleobases to it. UWA Opposition 1, 23:79-23. First, we read VO's disclosure to
8 teach making complementary AONs of various lengths:

9 The complementary regions are preferably designed such that,
10 when combined, they are specific for the exon in the pre-
11 mRNA. Such specificity may be created with various lengths
12 of complementary regions as this depends on the actual
13 sequences in other (pre-)mRNA in the system.

14 Ex. 1007, 3:12-17. Secondly, regardless of the presence or absence of an express
15 teaching in VO, one skilled in the art would have been motivated to modify the
16 sequence length of AONs to determine the optimum length for causing exon
17 skipping. Ex. 1012, ¶ 56.

18 UWA also argues that because of unpredictability of exon skipping there
19 would be no reasonable expectation of success in modifying the AONs length. We
20 recognize, as we detailed above, that there is a significant degree of
21 unpredictability in the effect of AON sequence length on the ability and efficiency
22 of an AON to cause exon skipping. However, those working in the art were also
23 aware that a degree of exon skipping capability would likely be maintained due to
24 a change in a small number of complementary nucleobases of an AON known to
25 cause skipping. As shown in Exhibit 2012, AONs hAON4 and hAON6, each are
26 taught to cause exon skipping. Ex. 2012, p. 1549, first column. hAON4 was
27 fifteen nucleobases long. hAON6 included the same fifteen bases but five
28 additional bases to the 5' end. Ex. 2012, Table 1. Thus, notwithstanding the

1 unpredictability, one having ordinary skill in the art would have had a reasonable
2 expectation of success that exon skipping would be maintained when a small
3 number of complementary nucleobases are added to h53AON1. It would have
4 been obvious, for example, to add the two complementary nucleobases dictated by
5 the known sequence of exon 53 to either end of h53AON1 with a reasonable
6 expectation that the resultant 20 base AON would cause exon skipping. See VO,
7 Ex. 1007, 9:28-30 (the AONs are preferably complementary to a consecutive part
8 of between 16 and 50 nucleotides of the exon RNA). Thus, Claims 1, 4, 19 and 20
9 are unpatentable under 35 U.S.C. § 103.

10 **B.**

11 *UWA Claims 5-18, 21-37, 41 and 43*

12 These claims depend, directly or indirectly from Claims 1 and 19. AZL
13 argues that the additional limitations of these claims do not present patentable
14 distinctions over Claims 1 and 19. AZL Motion 1, Paper 181, 15:25 – 27:10.

15 AZL identifies where the additional limitations are taught in VO, Kole,
16 Bennett or Latchman. *Id.* We have reviewed the teachings of these references and
17 concur that the additional limitations of Claims 5-18, 21-37, 41 and 43 are taught
18 in either VO or the other references and do not present patentable distinctions over
19 the cited art. UWA's opposition neither challenges that the references teach the
20 additional limitations nor that the add limitations present features that would
21 themselves render the subject matter of these claims nonobvious. UWA
22 Opposition 1, Paper 393, 28:5-13.

23 Claims 5-18, 21-37, 41 and 43 are unpatentable under 35 U.S.C. § 103(a).

24 **C.**

25 *UWA Claims 2, 3, 38-40, and 42.*

26 These claims depend from Claim 1. They require an AON “comprising SEQ
27 ID NO: 193” or “consisting of SEQ ID NO: 193.” Those AONs must also

1 “specifically hybridize[] to an exon 53 target region of the human dystrophin gene
2 inducing exon 53 skipping.” UWA Clean copy of Claims, Paper 12, Claim 1.
3 Thus these claims specify AONs that include at least the full 31 nucleobase
4 sequence of SEQ ID NO: 193.

5 **1.**

6 AZL argues that Claims 2 and 3 are anticipated by VO when considered
7 with the Koenig disclosures which disclose, *inter alia*, the full sequence of
8 Exon 53. AZL Motion 1, Paper 181, 3:17-4:6.

9 While we agree that one skilled in the art following VO’s teachings could
10 manufacture all the possible AONs that include the sequence of AZL’s SEQ ID
11 NO:29 and would be complementary to exon 53, we are not persuaded that one
12 skilled in the art would have a basis to reasonably expect that UWA’s minimum 31
13 nucleobase AONs would cause exon skipping. We held above that there is a
14 substantial degree of unpredictability in the exon skipping art especially with
15 respect of AON sequence length. When given the sequence of an AON which will
16 cause exon skipping, in this case the AON having the sequence of AZL’s SEQ ID
17 NO: 29, one skilled in the art would reasonably have expected that modifying that
18 sequence by adding or subtracting a relatively small number of exon 53
19 complementary nucleobases would likely maintain exon skipping. However,
20 AZL’s h53AON1 differs from UWA’s SEQ ID NO: 193 by thirteen nucleobases.
21 Because of the unpredictability of the effect of changing the AON sequence length
22 on exon skipping, we are not persuaded that VO’s teaching of h53AON1 would
23 put one having ordinary skill in the art in possession of the exon skipping AONs of
24 Claims 2 and 3. *Eli Lilly & Co. v. Zenith Goldline Pharms., Inc.*, 471 F.3d 1369,
25 1375 (Fed. Cir. 2006) (“To anticipate, a prior art reference must place the inventive
26 compound or composition in the possession of the public.”). Thus, we find that the

1 subject matter of Claims 2 and 3 has not been shown to be anticipated by
2 h53AON1.

3 **2.**

4 AZL also argues that the subject matter of the AONs of Claims 2 and 3, as
5 well as that of Claims 38-40 and 42, would have been obvious over VO. We are
6 not persuaded of the obviousness of the subject matter of those claims. As we
7 noted above, it is unpredictable whether exon skipping will be maintained when an
8 AON known to cause exon skipping is modified by changing the sequence length
9 but keeping complementarity. Because of the unpredictability, we are not
10 convinced that one ordinarily skilled in the art would have had a reasonable
11 expectation of success that an AON having the sequence of UWA's SEQ ID
12 NO: 193 would cause exon skipping.

13 **D.**

14 AZL's Motion 1 for a judgment that UWA Claims 1-43 are unpatentable
15 over certain prior art is granted as to Claims 1, 4-37, 41 and 43 and denied as to
16 Claims 2, 3, 38-40 and 42.

17 **VIII.**

18 *AZL Motion 2 to deny UWA the benefit of its Australian Application*

19 AZL moves to deny UWA the accorded benefit of the June 28, 2004, filing
20 date of its Australian application AU 2004903474. In AZL's view the Australian
21 application is not a constructive reduction to practice of the subject matter of the
22 count. We grant the motion.

23 The PTO's Interference Rules define "accord benefit" and "constructive
24 reduction to practice":

25 *Accord benefit* means Board recognition that a patent
26 application provides a proper constructive reduction to practice
27 under 35 U.S.C. § 102(g)(1).

1 *Constructive reduction to practice* means a described and
2 enabled anticipation under 35 U.S.C. § 102(g)(1), in a patent
3 application of the subject matter of a count.

4 37 C.F.R. § 41.200. *See Hunt v. Treppschuh*, 523 F.2d1386,1389 (Fed. Circ.
5 1975). (For the purpose of priority in an interference, where a “parent application
6 is relied upon as a prior constructive reduction to practice[,] . . . the § 112, first
7 paragraph requirements need only be met for an *embodiment* within the count.”)
8 *See also, Falkner*, 448 F.3d at 1362.

9 The count of this interference is:

10 Claim 15 of [AZL] Application 11/233,495 or Claim 1 of
11 [UWA] Patent 8,455,636.

12 Declaration, Paper 1, p. 4. The claims of the count are reproduced below:

13 AZL Claim 15:

14 An isolated antisense oligonucleotide of
15 [1] 15 to 80 nucleotides comprising
16 [a] at least 15 bases of the sequence
17 cuguugccuccgguucug (SEQ ID NO: 29),
18 [2] wherein said oligonucleotide induces exon 53 skipping in
19 the human dystrophin pre-mRNA,
20 [3] said oligonucleotide comprising a modification selected
21 from the group consisting of:
22 [a] 2'-O -methyl,
23 [b] 2'-O-methyl- phosphorothioate,
24 [c] a morpholine ring,
25 [d] a phosphorodiamidate linkage,
26 [e] a peptide nucleic acid and
27 [f] a locked nucleic acid.

28 AZL Clean Copy of Claims, Paper 8, 1:3-8.

29 UWA Claim 1:

30 An isolated antisense oligonucleotide of
31 [1] 20 to 50 nucleotides in length comprising
32 [a] at least 20 consecutive nucleotides of SEQ ID
33 NO:193,

1 [2] wherein the oligonucleotide specifically hybridizes to an
2 exon 53 target region of the human dystrophin gene
3 inducing exon 53 skipping, and
4 [3] wherein the uracil bases are optionally thymine bases.

5 UWA Clean Copy of Claims, Paper 12, APP A-1.

6 AZL argues that the Australian application neither discloses an AON that is
7 said to cause skipping of exon 53 nor a sequence that meets the limitations of the
8 claims that make up the count. AZL Motion 2, Paper 26, 15:13 – 17:16. UWA
9 does not argue to the contrary. Rather it argues that the motion should not be
10 reached. Since benefit is determined based upon the count and the count may
11 change as a result of the decisions on motion, UWA says that it is premature to
12 reach the motion at this time. UWA Opposition 2, Paper 394, 1:8 – 2:15.

13 We have reviewed UWA's Australian application and concur with AZL that
14 there is no description of exon 53 skipping or of an AON that would otherwise
15 meet the limitations of the count. The Australian application does not provide any
16 description of exon 53 skipping. That application could not provide a constructive
17 reduction to practice for any count directed to exon 53 skipping that could be
18 formulated.

19 AZL's Motion 2 to deny UWA the benefit of Australian Application
20 AU 2004903474 is granted. This interference will be redeclared to conform the
21 accorded benefit to our decision.

22 **IX.**

23 *AZL's Motion 3 for unpatentability under 35 U.S.C. § 101*

24 AZL's Motion 3 requests a judgment that UWA's Claims 1-4, 19-21, and
25 36-42 are unpatentable under 35 U.S.C. § 101 in view of *Association for*
26 *Molecular Pathology v. Myriad Genetics, Inc.*, ___ U.S. ___, 133 S.Ct. 2107

(2013). Paper 27. Because those claims cover naturally occurring DNA and are not limited to a new application of exon 53 information, we grant the motion.

UWA's involved claims are directed to AONs of 20-50 nucleobases that hybridizes to a region of exon 53 of the human dystrophin gene causing exon 53 to be skipped. The AON must include at least 20 consecutive nucleobases of UWA's SEQ ID NO: 193. Additionally, the uracil bases may be substituted with thymine. We reproduce representative Claim 1 below (with paragraphing added):

1. An isolated antisense oligonucleotide
of 20 to 50 nucleotides in length comprising
at least 20 consecutive nucleotides of SEQ ID NO:193,
wherein the oligonucleotide specifically hybridizes to an exon
53 target region of the human dystrophin gene
inducing exon 53 skipping, and
wherein the uracil bases are optionally thymine bases.

UWA Clean Copy of Claims, Paper 8, App A-1, Claim 1.

A.

According to AZL, the Claims 1-4, 19-21 and 36-42 are unpatentable under § 101 because they cover naturally occurring DNA sequences. AZL points out that the DNA version of the AON covered by Claim 1 is identical to sequences present in exon 53 of the dystrophin gene. AZL Motion 3, Paper 27, 5:13 – 6:6.

UWA does not argue that that is incorrect. Rather, UWA argues that the claimed AONs differ in structure, properties, and functionally from anything existing in nature. UWA argues that claimed AONs are a subset of the antisense strand of exon 53 DNA. UWA Opposition 3, 6:6 – 7:13; 7:23 – 9:21. It also argues that each AON will have different binding characteristics. UWA Opposition 3, 6:6 – 7:13.

Myriad effectively answers these points. Myriad's Claim 5 and 6 were directed to a subset of the BRCA1 and BRCA2 DNA—15 or more nucleotides that

1 encoded the BRCA1 and BRCA2 polypeptides. Those shorter DNA segments
2 were also a subset of the BRCA1 and BRCA2 genes and would necessarily have
3 different properties due to their shorter length. Yet the subject matter of Myriad’s
4 Claims 5 and 6 was not considered patent eligible. Myriad, 133 S.Ct. at 2113 and
5 2118 (discussion of Claim 5 and discussion of isolating DNA). Similar to the
6 situation in *Myriad*, UWA’s challenged claims would give it the exclusive right to
7 isolate any strand of 20 to 50 nucleotides that include at least 20 consecutive
8 nucleotides of SEQ ID NO: 193.

9 We are not persuaded that the cited “functionality” makes any difference as
10 to UWA’s claims other than to identify a property of the AONs. A new
11 application of knowledge about exon 53 and its sequence might tip the balance in
12 favor of eligibility. As noted in *Myriad*:

13 It is important to note what is not implicated by this decision.

14 * * * * *

15 [T]his case does not involve patents on new applications of
16 knowledge about the BRCA1 and BRCA2 genes. Judge Bryson
17 aptly noted that, “[a]s the first party with knowledge of the
18 [BRCA1 and BRCA2] sequences, Myriad was in an excellent
19 position to claim applications of that knowledge. Many of its
20 unchallenged claims are limited to such applications.

21 *Myriad*, 133 S.Ct. at 2120. None of UWA’s challenged claims appear to be
22 limited to the application of exon skipping. In UWA’s claims exon skipping
23 defines a characteristic of the AONs, the recitation of that characteristic, however,
24 does not limit the application of the claimed AONs to any particular use or
25 function. The Federal Circuit’s holding in *In re BRCA1- and BRCA2-Based*
26 *Hereditary Cancer Test Patent Litig.*, 774 F.3d 755 (Fed. Cir. 2014) referred to in
27 UWA’s Opposition 3 (Paper 395, 11:17-12:2) is not inconsistent with the Supreme
28 Court’s statement quoted above as to claims limited to new applications of the
29 knowledge about the gene (DNA) as compared to claims directed to and

1 controlling naturally occurring sequences. UWA Claims 1-4, 19-21 and 37-42
2 have not be shown to have a unique structure, different from anything found in
3 nature. *BRCA1- and BRCA2*, 774 F.3 at 761.

4 UWA argues that the claims are directed to significantly more than naturally
5 occurring DNA. UWA Opposition 3, 10:1-28. Specifically, UWA argues that the
6 claimed subject matter effects “transformation or reduction of a particular article to
7 a different state or thing.” However, none of challenged claims are directed to a
8 method. UWA Claims 1-4, 19-21 and 36-42 do not require that anything be
9 transformed.

10 Claims 36 and 39-42 are directed to pharmaceutical compositions
11 comprising AONs of Claims 1-3, 19 or 38 and adding a “saline solution including
12 a phosphate buffer” AZL argues that the limitations represent well-understood,
13 routine or conventional additions to pharmaceutical compositions. Such additions
14 are said not to transform a naturally occurring product into a patent eligible
15 application of the AON. UWA Motion 3, Paper 27,

16 UWA does not challenge this argument. We have reviewed the evidence
17 cited by AZL and agree that the additions are represent well-understood, routine or
18 matters conventional in the art and do not transform Claims 36 and 39-42 into
19 patent eligible subject matter.

20 AZL’s motion for a judgment that UWA’s Claims 1-4, 19-21, and 37-42 are
21 unpatentable under 35 U.S.C. § 101 in view of *Myriad* is granted.

22 **X.**

23 *AZL Responsive Motion 4 to add two additional claims*

24 AZL moves to add additional claims 104 and 105 in response to UWA’s
25 assertions of unpatentability in UWA Motions 1 and 2. Paper 241. We deny the
26 motion. Proposed claim 104 would be unpatentable under 35 U.S.C. § 112 for the
27 same reasons we stated with respect to AZL Claims 15, 76, 78-80, 82, 84, 86, 88-

90, 97, 98, and 100-103. Claim 105 is unnecessary in light of our holding that claim 77 was not proved to be unpatentable.

The two claims suggested by AZL are reproduced below:

104. **(New)** An oligonucleotide of between 14 and 50 nucleotides comprising the sequence of h53AON1 (SEQ ID NO: 29), wherein said oligonucleotide comprises a 2'-O-methyl-phosphorothioate oligonucleotide modification.

105. **(New)** An isolated antisense oligonucleotide, wherein the oligonucleotide is 18 nucleotides and comprises the sequence cuguugccuuccgguucug (SEQ ID NO: 29), wherein each internal nucleoside linkage of the oligonucleotide is a phosphorothioate linkage.

AZL Motion 4, Paper 241, 1:8-15.

Proposed Claim 104

A party moving to add a claim must show it is patentable. 37 CFR § 41.208(c). We held above that AZL's Claims 15, 76, 78-80, 82, 84, 86, 88-90, 97, 98, and 100-103 were unpatentable under § 112(a). Each of those claims recited a range of nucleobases. We held that those claims were unpatentable because the person having ordinary skill in the art would not have been put in possession of the full range of AON sequence lengths specified in those claims. One of those claims, for example, specified sequence lengths of 18-50 nucleobases. AZL Clean Copy of Claims, Paper 8, Claim 78. The broader scope of "between 14 and 50" nucleobases of proposed claim 104 would be unpatentable for the reasons stated with respect to claims 15, 76, 78-80, 82, 84, 86, 88-90, 97, 98, and 100-103.

Proposed Claim 105

Claim 105 is similar to claim 77 substituting phosphorothioate linkage for the Markush group of 2'-O-methyl, 2'-O-methyl-phosphorothioate, a morpholine ring, a phosphorodiamidate linkage, a peptide nucleic acid and a locked nucleic

1 acid. Because we held claim 77 was not shown to be unpatentable, we are not
2 persuaded that the amendment is necessary to cure a defect raised by UWA's
3 motions. 37 C.F.R. § 41,121(a)(2). In any event, as an applicant, AZL may seek
4 entry of the amendment when the application returns to the jurisdiction of the
5 patent examiner.

6 **XI.**

7 As a result of the decisions on AZL's Motions 1 and 3 all of Junior Party
8 UWA's claims have been held unpatentable. As UWA has not alleged a date of
9 invention earlier than AZL's accorded benefit date of March 21, 2003, (UWA
10 Priority Statement, Paper 214) there is no apparent reason to continue this
11 interference and proceed to the priority phase. Accordingly, a judgment will be
12 issued in a separate paper.

13 **SUMMARY**

14 We dismiss UWA's Motion 4 to exclude Exhibits 1012, 1067 and 1186 and
15 deny that motion with respect paragraphs 4 to 18 of Exhibit 1125.

16 We grant UWA's Motion 1 for unpatentability under 35 U.S.C. § 112(a)
17 with respect to AZL Claims 15, 76, 78-80, 82, 84, 86, 88-90, 97, 98, and 100-103,
18 but deny it with respect to AZL Claim 77.

19 We deny UWA's Motion 2 that AZL's Claims 15, 76-80, 82, 84, 86, 88-90,
20 97, 98, and 100-103 are indefinite under 35 U.S.C. § 112(b).

21 We deny UWA's Motion 3 to declare an additional interference.

22 We grant AZL's Motion 1 that UWA's claims are unpatentable over prior
23 art with respect to Claims 1, 4-38 and 41-43, but deny it as to Claims 2, 3, 38-40,
24 and 42.

25 We grant AZL's Motion 3 asserting that UWA's Claims 1-4, 19-21 and 37-
26 42 are not directed to patent eligible subject matter under 35 U.S.C. § 101.

1 We grant AZL's Motion 2 to deny UWA the benefit of the filing date of
2 Australian Application AU 2004903474.

3 We deny AZL's Motion 4 to add two additional claims to its involved
4 application.

5 Because all of UWA's claims have been held to be unpatentable, and UWA
6 does not assert a date earlier than AZL's accorded benefit date, there is no apparent
7 reason to proceed to a determination of priority. A judgment will be entered in a
8 separate paper.

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EXHIBIT 18

The Complete Sequence of Dystrophin Predicts a Rod-Shaped Cytoskeletal Protein

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Summary

The complete sequence of the human Duchenne muscular dystrophy (DMD) cDNA has been determined. The 3685 encoded amino acids of the protein product, dystrophin, can be separated into four domains. The 240 amino acid N-terminal domain has been shown to be conserved with the actin-binding domain of α -actinin. A large second domain is predicted to be rod-shaped and formed by the succession of 25 triple-helical segments similar to the repeat domains of spectrin. The repeat segment is followed by a cysteine-rich segment that is similar in part to the entire COOH domain of the Dictyostelium α -actinin, while the 420 amino acid C-terminal domain of dystrophin does not show any similarity to previously reported proteins. The functional significance of some of the domains is addressed relative to the phenotypic characteristics of some Becker muscular dystrophy patients. Dystrophin shares many features with the cytoskeletal protein spectrin and α -actinin and is a large structural protein that is likely to adopt a rod shape about 150 nm in length.

Introduction

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are both human X-linked degenerative disorders of muscle. DMD affects about 1 in 3500 live born males and results in a fatal evolution before the end of the third decade of life, while BMD is less severe and less frequent (Moser, 1984). Until recently, the molecular and biochemical basis of DMD and BMD was unknown. Cytogenetically detectable alterations of the DMD/BMD locus (Verellen-Dumoulin et al., 1984; Francke et al., 1985) initiated the isolation of the gene which, when defective, results in Duchenne or Becker muscular dystrophy (Monaco et al., 1986; Koenig et al., 1987; Burghes et al., 1987). Portions of the coding sequence of the gene (cDNA) have been used to establish the muscle specificity of expression of the gene (Hoffman et al., 1987a; Lev et al., 1987) and to produce polyclonal antisera directed against the encoded protein product (Hoffman et al., 1987b). The an-

tisera detect a protein in muscle, called dystrophin, that is about 400 kd in size and which has been shown to be associated the transverse tubules of the muscle triadic structures (Hoffman et al., 1987c; Knudson et al., 1988). The absence of dystrophin in DMD muscle and its altered size in BMD muscle (Hoffman et al., 1988) establishes the disruption of dystrophin as the primary biochemical defect in Duchenne/Becker muscular dystrophy. From partial sequence information on dystrophin (Koenig et al., 1987; Hoffman et al., 1987a), the N-terminal 240 amino acids of dystrophin have been shown to be homologous to the N-terminus of α -actinin (Hammond, 1987) which corresponds to a presumed actin-binding domain (Baron et al., 1987; Noegel et al., 1987), and the subsequent 1200 amino acids have been shown to exhibit weak repeats similar to those found in spectrin and α -actinin (Davison and Critchley, 1988).

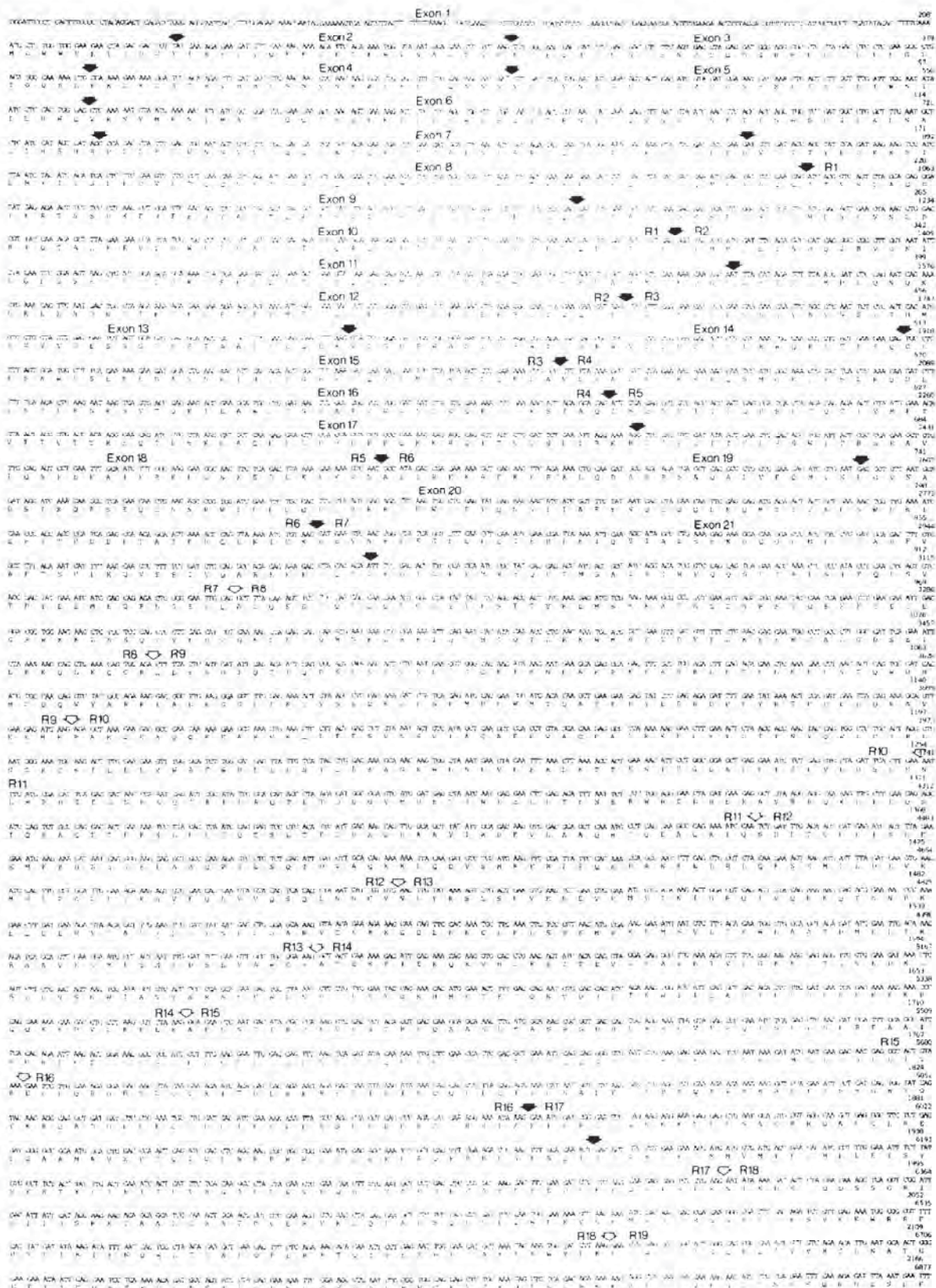
In this paper, we report the entire DMD cDNA sequence and the deduced primary structure of dystrophin. We use the amino acid sequence to define different domains of the protein and to predict a preliminary structure by comparing dystrophin with spectrin and α -actinin.

Results

Complete Nucleotide Sequence of the DMD cDNA and Preliminary Exon Organization

The entire sequence of the human DMD cDNA is presented in Figure 1. The 14 kb sequence is derived from 22 independent overlapping cDNA clones (Koenig et al., 1987). For the majority of the sequence, the sequences of the two complementary strands were determined from two different clones. Four cDNA clones appeared to differ from the other cDNA sequence by restriction mapping and/or sequence determination. In two cases, sequence divergence occurred at sites perfectly matching the consensus splice sites (Mount, 1982). The diverging sequences have stop codons in all three reading frames and are presumed to correspond to intron sequences. The presumed intron sequences were found in both cases on the 3' end of the cDNA clones, and in one case a full 1 kb intron was included in the cDNA clone followed by an exon and the beginning of the next intron. These two cDNA clones probably represent hnRNA forms of the DMD transcript with the first introns of the gene already excised. The finding of cDNAs representing hnRNA forms may be correlated to the complexity of the DMD gene, which is formed by over 60 exons (Koenig et al., 1987). The two other cDNA clones showed minor structural differences that seemed to be artifacts of cloning rather than alternative splicing.

In addition to completing the cDNA sequence, the sequence of the intron-exon boundaries were determined for exons mapping from the 5' end of the gene to the end of the DSX164 locus (see Experimental Procedures). The limits of the first 21 exons were determined, and they are presented in Figure 1. The sequence of the cloned



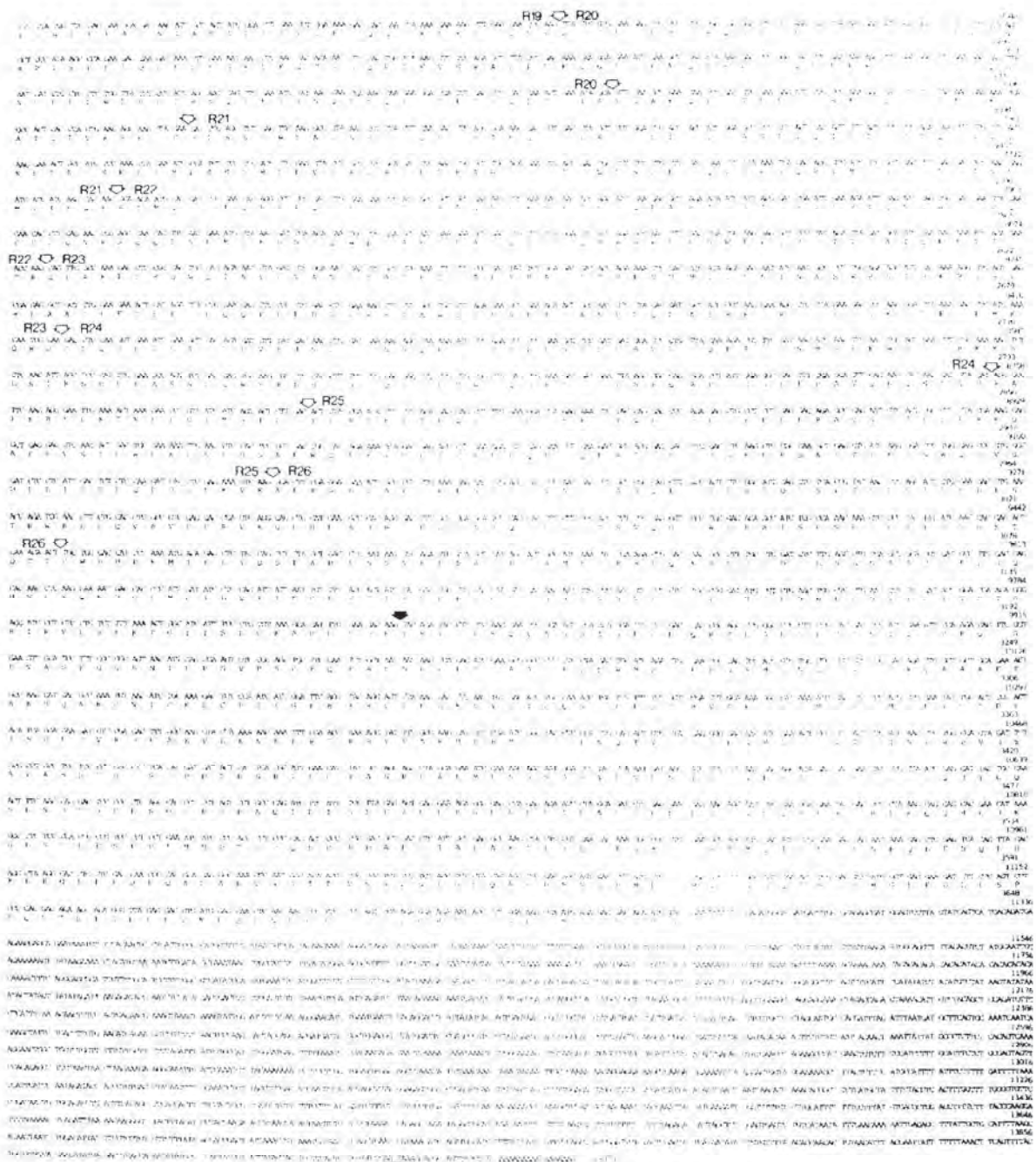
The Primary Structure of Dystrophin
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Figure 1. Nucleotide Sequence of the DMD transcript and Deduced Primary Structure of the Encoded Protein

The DNA sequence was determined as described in Experimental Procedures and used to predict the protein sequence. The exon borders, where known, are indicated by black arrows, and the predicted repeat borders are indicated by light arrows. The three exon borders that are beyond nucleotide 5000 have been determined from the sequence of abnormal cDNA (see Results). The exons are numbered in the middle of the exon (Exon #), and the repeats are numbered at the beginning and at the end of the repeats (R#). The polyadenylation consensus sequence (Proudfoot and Brownlee, 1976) found at the end of the transcript is underlined. The GenBank accession number for this sequence is M18533.

genomic exons matched that found for the cDNA sequence with one exception. The published 5' end of the cDNA GGGATCCCC (Koenig et al., 1987) was found to be GGGATCAC in the corresponding genomic sequence.

The Amino Acid Sequence of the DMD Gene Product

The DNA sequence was examined for presence of open reading frames consistent with the partial open reading frame published in Hoffman et al. (1987a) and Koenig et al.

Cell
222

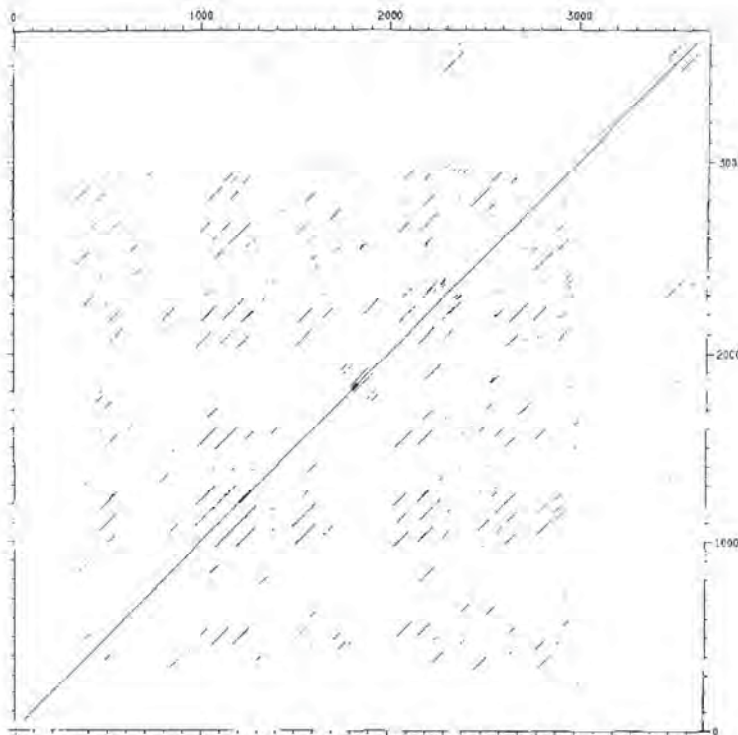


Figure 2. Dot Plot of the Dystrophin Amino Acid Sequence.

The predicted amino acid sequence for the long open reading frame was analyzed for similar sequences within itself by the computer program COMPARE with a stringency of 39 and a window length of 100 (see Experimental Procedures). The output of this comparison was plotted by DOTPLOT (see Experimental Procedures) and is shown. The short lines parallel to the central diagonal represent internal similarities. The axes are labeled in residue numbers.

(1987). There is a single very large open reading frame (Figure 1) that follows the ATG initiation codon (Koenig et al., 1987) and extends over 11 kb of the cDNA. The stop codon of this open reading frame is followed by a 2.7 kb segment with no apparent coding potential. The open reading frame encodes a protein 3685 amino acids long, with a predicted molecular weight of 427 kd, in accordance with the reported 400 kd size of dystrophin, the protein product of the DMD gene (Hoffman et al., 1987b). The hydropathicity index calculated according to Kyte and Doolittle (1982) reveals that dystrophin is predominantly hydrophilic throughout the entire molecule. Thirty-one percent of amino acids are charged (Asp, Glu, His, Lys, and Arg). No substantial hydrophobic stretch of amino acids was found that could account for a signal peptide segment or a membrane spanning segment. The Chou and Fasman (1978) prediction of secondary structure reveals a very high potential for α -helical formation over the majority of the sequence. These α -helices, however, are frequently interrupted by prolines. The analysis of the cysteine distribution along the molecule reveals that the segment from amino acid positions 2200 to 3080 is completely devoid of cysteines; this segment is immediately followed by a relatively cysteine-rich segment (15 cysteines in 280 amino acids). The latter segment will be referred to as the "cysteine-rich" segment in the text that follows. The entire DMD cDNA and protein sequences were used to search for similarity with other sequences available from the GenBank nucleic acid data base and the PIR protein data base. Only the previously reported strong similarity to the nonmuscle α -actinin of chicken (Hammond, 1987) was

found to be statistically significant. Dystrophin and α -actinin share the greatest similarity over a putative actin-binding N-terminal region that corresponds to about amino acids 14 to 240 of dystrophin (Hammond, 1987, and data not shown). This domain covers exons 2 to 8 while the first exon comprises the entire 5' untranslated sequence and encodes for the first 11 amino acids of the protein.

Repeated Domains in Dystrophin

Recently, Davison and Crichtley (1987) described the occurrence of weak repeats similar to those found in spectrin and α -actinin in the published partial DMD sequences. With the complete protein sequence, we further analyzed the extent of these repeats along the molecule and further characterized their nature. The dot matrix plot of the entire amino acid sequence against itself revealed many small diagonals spaced by about 100 amino acids and extending approximately from positions 300 to 3000 (Figure 2). The dot matrix gave a rough determination of the limits of the repeats, which were then aligned 2 by 2 with BESTFIT (UWGCG). The similarity between most repeats ranged from 10% to 25% identical matches. Positioning the determined exon borders on the aligned repeats shows that the 3' splice site of exons 10, 12, 15, 18, and 20 correspond to the same position within the repeat. In addition, all five splice sites occur at the end of the coding triplet. We assumed that these exon borders corresponded to the limits of a genetic unit that was duplicated, and we used them to define the limits of the first six repeats. With these limits, repeats 1, 2, 5, and 6 are formed by two exons while repeat 3 is formed by three exons. Repeat 4 is a truncated repeat

The Primary Structure of Dystrophin 223

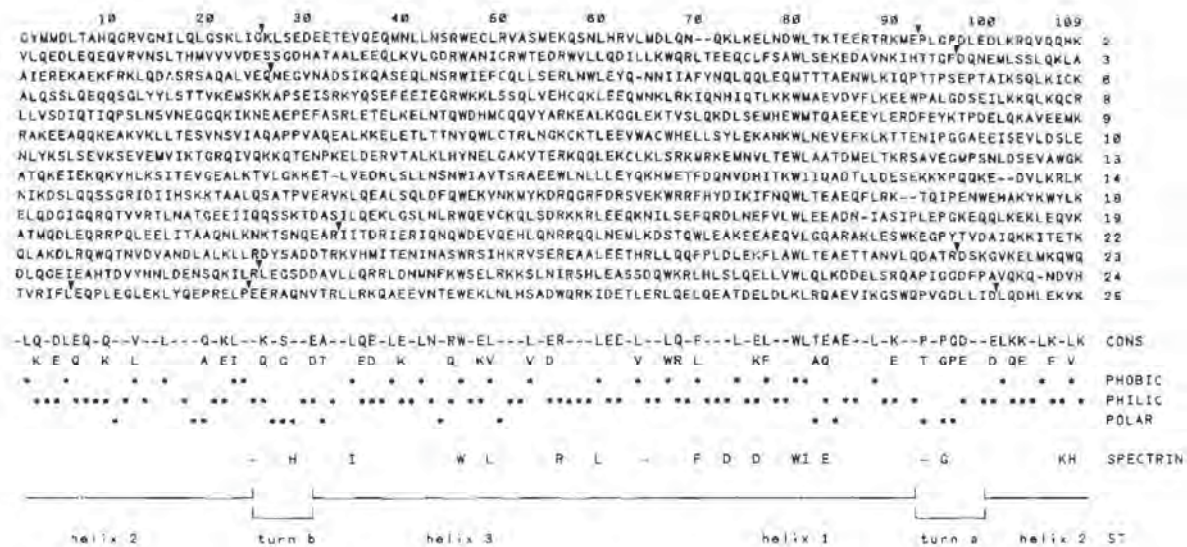


Figure 3. Table of 14 Dystrophin Repeats and Alignment with the Spectrin Repeat Consensus

The optimal alignment of the 14 repeats that exhibit the most internal similarities on the dot plot of Figure 2 is shown. The repeat number is given to the right. Dashes and dark arrowheads indicate insertions and deletions, respectively, which allow optimized alignment. Arrowheads represent insertions of one or two residues with the exception of the six-residue insertion of repeat 23. The beginning and the end of repeats 2, 3, and 6 correspond to established exon borders. CONS: the consensus sequence derived from the 14 repeats is based on the presence of one or two amino acids at a given position within a repeat $\geq 50\%$ of the time ($\geq 7/14$). PHOBIC, PHILIC, and POLAR: a broader consensus was derived (as in CONS) with the amino acids grouped into three classes: PHOBIC, the large hydrophobic and aromatic residues (Leu, Ile, Val, Met, Phe, Tyr, and Trp); PHILIC, the charged and highly hydrophilic residues (Asp, Glu, Asn, Gln, His, Lys, and Arg); POLAR, the small polar residues (Gly, Ala, Ser, Thr, Cys, and Pro). SPECTRIN, the 15 best conserved positions of the spectrin consensus (according to Speicher and Marchesi, 1984) have been aligned with the dystrophin consensus. The three dashes indicate the approximate position of the gaps that should be inserted to align the spectrin consensus. ST: predicted secondary structure of the repeats. It is interesting to notice that the gaps introduced to align the dystrophin repeats as well as the spectrin repeat consensus occur predominantly in or near both turn regions.

(60 amino acids) and is formed by only one exon. The exon borders that fall in the middle of a repeat are not found at similar positions within the repeat. The limits of the remaining repeats can be predicted from their alignment with repeats 1 to 6, and their start and end points are indicated upon the sequence presented in Figure 1. The alignment for the less conserved repeats is still tentative.

The 14 most similar repeats were used to derive a consensus sequence (Figure 3). The consensus sequence is 109 amino acids long with one or two predominantly occurring amino acids (present in 7 or more repeats out of 14) assignable for 60 positions. The most striking feature of the consensus is the conservation of the tryptophan residues at positions 46 and 80. There is a clustering of proline and glycine between positions 28–29 and positions 92–98, indicative of the occurrence of turns in these two parts of the repeat. A less stringent consensus could also be derived by grouping the amino acids into three classes: the hydrophobic amino acids, the small polar amino acids, and the charged and related amino acids (see legend to Figure 3). An amino acid class could be assigned for 99 positions. This grouping reveals the alternation of one hydrophobic position with two or three hydrophilic positions, indicative of heptad repeats (McLachlan and Karn, 1982), extending between the two potential turn positions (see bottom of Figure 3). The occurrence of multiple heptad repeats (noticed in Hoffman et al., 1987a) strongly predicts that these regions adopt an α -helical

conformation and indicates that the α -helices may be involved in coiled-coil interactions (McLachlan and Karn, 1982).

The entire dystrophin coding sequence contains 26 repeat domains organized in tandem. They extend from the end of the NH₂ "actin-binding" domain to the beginning of the cysteine-rich domain (a schematic of the organization of the domains and repeats of dystrophin is presented in Figure 4). The length of the repeats ranges between 88 and 126 amino acids. Two segments (between repeats 20–21, 33 amino acids, and between repeats 25–26, 19 amino acids) were too short to be aligned with other repeats. The degree of similarity is also variable from one repeat to the other, as can be seen from the dot matrix comparison.

Analysis of the C-Terminal Domain of Dystrophin

Beyond the repeat domains, dystrophin is formed by a relatively cysteine-rich segment extending from amino acid position 3080 to 3360 followed by 325 additional amino acids with only one cysteine residue. To further investigate the extent of similarity between dystrophin and the α -actinins, the C-terminus of both proteins were compared (the C-terminal sequence of spectrin was not available for comparison). A significant similarity is found between the entire C-terminal domain of the slime mold (*Dictyostelium discoideum*) α -actinin, which contains two potential Ca²⁺-binding sites (Noegel et al., 1987), and 150 amino acids

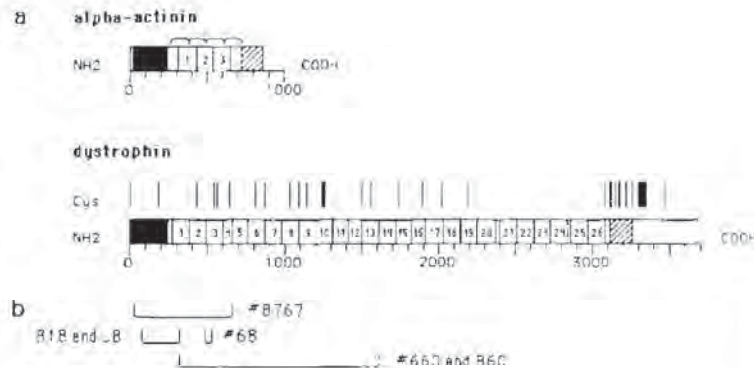
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Figure 4 Domain Organization of Dystrophin and α -Actinin

(a) Landmarks of the predicted amino acid structure of dystrophin and the slime mold α -actinin are diagrammatically represented. Black box: putative actin-binding domain. Numbered open boxes: repeat units. The organization of the four triple helical domains (see Discussion) of α -actinin is represented by the brackets on top of the repeat boxes. Striped box: putative EF hand Ca^{2+} -binding sites of α -actinin and homologous segment of dystrophin. The position of the cysteine residues of dystrophin is indicated by vertical bars on top of the dystrophin diagram. The scale in amino acid residues is given for both proteins.

(b) The extent of the internal protein deletions of the six Becker patients discussed in the text is shown along the dystrophin diagram. Patient #8767 is missing exons 3 to 16, patients JB and B18 are missing exons 5 to 9, patient #68 is missing exon 13, and the deletions of patients #660 and B60 extend from exon 10 (included) to beyond exon 21.

of the dystrophin cysteine-rich domain (Figures 4 and 5). Two gaps were introduced to optimize alignment, and there are 24% identical matches over 142 aligned amino acids (Figure 5). For comparison, the similarity between the C-terminal domains of the Dictyostelium α -actinin and the chicken fibroblastic α -actinin (Baron et al., 1987) is 31% of identical matches (Figure 5). In contrast, dystrophin and the chicken α -actinin are much less conserved over that segment (13% identical matches), suggesting that the Dictyostelium α -actinin segment may represent an ancestral sequence from which both dystrophin and the chicken α -actinin have evolved. The dystrophin segment does not match well with the EF-hand Ca^{2+} -binding site consensus (Tufty and Kretsinger, 1975), but might still have conserved the loop structure of the EF-hand.

The last 420 amino acids of dystrophin did not exhibit any similarity to α -actinin. This domain was used again to search for similarities in the GenBank (release 54) and PIR (release 14) data bases. The use of a limited section of dystrophin lowers the level of nonspecific matches and should allow the detection of less conserved sequences. Lowering the level of nonspecific matches did not reveal any new similarity.

Comparison of Domain Deletions with the Dystrophic Phenotype

Analysis of deletions of limited portions of dystrophin may give some insight into the functional significance of the various domains of the protein. Ideally, the high incidence of deletions of the DMD gene in independent patients should facilitate this study of domain function. However, only the deletions that do not result in a frame shift of the coding sequence represent true internal protein deletions. Some, if not most, of these internal protein deletions result in the less severe Becker muscular dystrophy phenotype, while deletions that disrupt the reading frame usually result in the Duchenne muscular dystrophy phe-

notype (Monaco et al., 1988). We discuss here the five Becker patients with genomic deletions that have been described in Monaco et al. (1988) with the addition of another Becker patient, #8767, whose deletion was first detected by Dr. B. deMartinville (University of Minnesota, Minneapolis) and further analyzed in our laboratory (see Experimental Procedures). The extent of the deletions is shown along the dystrophin diagram in Figure 4. The deletions of four of these patients have been precisely defined and the two exons flanking each of the deletions were shown to maintain an open reading frame. Because the reading frame is uninterrupted, one would predict a lower molecular weight, presumably semifunctional, dystrophin protein to be produced.

Three Becker patients have deletions that remove only sequences coding for repeat domains. The first patient (#68) has little functional deficits at age 17 and is missing only the first third of repeat 3. The two other Becker patients (#660 and B60) have deletions that remove repeats 1 to 12 or 13. The clinical progression of patients 660 and B60 is very different; one has been confined to a wheelchair since age 16 and the other can still walk at age 25. A more precise definition of the distal breakpoint of the two deletions will allow us to address the molecular basis of the phenotypic differences. Three Becker patients have deletions involving the N-terminal "actin-binding" domain of dystrophin. Two unrelated patients (B18 and JB) have similar deletions, with the same exons deleted, resulting in a loss of the last two-thirds of the "actin-binding" domain, and they both showed significant muscle weakness before age 16 (see Experimental Procedures). Finally, the dystrophin of patient #8767 is missing repeats 1, 2, 3, and 4 but also almost the complete "actin-binding" domain (with the exception of the first 18 amino acids of that domain). The deletion of patient #8767 encompasses the deletion of patients B18 and JB, yet his clinical evolution is significantly milder, since he can still walk at age 29.

The Primary Structure of Dystrophin 225

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DMD 3115 LQKALCLDLLSLAACDALDQHNLKQNDQPMIDILQIINCLTTIYDRLEQEHNNLYNPLCYDMCLNWLNNVYDTGRTGRIRVLSFKTGISLCKA-H_ 3211
ADD 720 LAKQLTGVTAEELSEFKACFSHFQKDNKLNRLFEFSCLKSIGDELTEE-----QLNQVISKIDTDGNGTISFEEDICVMVSSRHGTDG 804
ACHK 738 LTRDAKGISQEQWNEFRASFNFDRKKTGMWDCEDFRACLISMGYNWGEA-----EFARIMSIVDPNRMGVVTFQAFIDFWSETADTD 828

      EL' LL' LD'D'DG ID' EL' LL' L'      EL' LL' LD'D'DG ID' EL' LL' L'
      X Y Z-Y-X -Z      X Y Z-Y-X -Z

DMD 3212 EDKYRYLFKQVASSTGFCQRRRLGLLLHOSIQIPRLGEVA-----SFGGSNIEPSVRSCEFQ 3268
ADD 805 VESTKAAFKVMAEOKDFITEAQIRAAISDSKQIDYLLASMP-----AVEGGFDYNSFAEKLYQ 862 COOH END
ACHK 821 ADQVMASFKI_AGDKNYITVDELRLRFPDQAEYCIARMAPYNGRDVAVPGALDYMSFSTALYGESDL 887 COOH END

```

Figure 5 Similarity between the Two Potential EF-Hand Ca^{2+} -Binding Sites of the Slime Mold α -Actinin and a Corresponding Segment of Dystrophin. The sequences were aligned with the aid of the computer program BESTFIT (UWGCG, see Experimental Procedures). The alignment of the slime mold α -actinin (Noegel et al., 1987) with the chicken α -actinin (Baron et al., 1987) is shown for comparison. Stars indicate identical matches and points indicate conservative matches. DMD, dystrophin; ADD, α -actinin of Dictyostelium discoideum; ACHK, chicken α -actinin. Below the chicken α -actinin sequence are the EF-hand consensus (Tufty and Kretsinger, 1975; D', oxygen-containing side chain; L', hydrophobic side chain), and the positions of the Ca^{2+} -chelating side chains (X, Y, Z; X-Z).

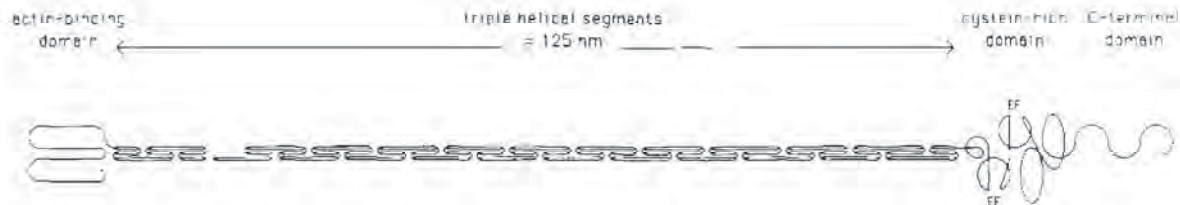


Figure 6. A Model of the Dystrophin Molecule

Four domains of dystrophin are represented on this tentative schematic. The conformation of the "actin-binding," cysteine-rich, and C-terminal domains is purely speculative, and they are represented as broad loops simply to illustrate that they may adopt a more globular structure than the rod section. The rod section is formed by 25 triple helical segments. A triple helical segment is formed by the last half of a repeat and the first half of the following repeat. The fourth triple helical segment is only formed by the first half of repeat 5 since repeat 4 is truncated (see text). The 20th and 24th triple helical segments are represented longer simply to indicate the existence of short segments between repeats 20 and 21 and between repeats 24 and 25. EF indicates two loops that might have conserved an EF-hand conformation (see text).

Discussion

Predicted Structure of Dystrophin

Dystrophin appears to be formed by four different domains: an N-terminal "actin-binding" domain (Hammond, 1987), a middle domain formed by 26 repeats in tandem, a cysteine-rich domain, and a less characterized COOH domain. The first three domains show significant similarity to the three domains of α -actinin while the C-terminal domain did not reveal any similarity to other known proteins. The repeats of the second domain were shown to be similar to the repeats found in spectrin and α -actinin, and in particular, the two characteristic tryptophan positions are found in all three types of repeat (Davison and Critchley, 1988). The 109 amino acid repeat consensus of dystrophin can be aligned with the 106 amino acid repeat consensus of spectrin (Speicher and Marchesi, 1984) by the insertion of three single amino acid gaps (see bottom of Figure 3), while the 120 amino acid repeat consensus of α -actinin (Baron et al., 1987) seems more distantly related. We found that the two clusters of proline and glycine and nearly all hydrophobic positions of the dystrophin consensus are also conserved in the consensus of spectrin and α -actinin (data not shown). The predicted secondary structure of the dystrophin repeats is similar to the pre-

dicted structure of the spectrin repeats (Speicher and Marchesi, 1984), with the exception of the α -helix extending from position 31 to 92 that was predicted to be interrupted in spectrin by a median "connecting region." A triple helical model has been proposed for the spectrin repeats (Speicher et al., 1983; Speicher and Marchesi, 1984) based on sequence data. In addition, the length of a repeat unit (about 5 nm, as deduced from electron microscope measurement) is in good agreement with the triple helical model since this length is a third of the length of a single α -helix with the same number of amino acids (Speicher et al., 1983). This model is likely to be valid for the dystrophin and α -actinin repeats. The model predicts that a triple helical segment is formed by the juxtaposition of the three α -helices 1, 2, and 3 that are connected by turns a and b (see bottom of Figure 3). The dystrophin triple helical segment would then be formed by the last half of a repeat and the first half of the following repeat. In agreement with the model, the half-repeats that do not interact with another half-repeat (first half of repeat 1, last half of repeat 26, and the half-repeat following the truncated repeat 4) show very poor similarity to the repeat consensus.

The middle domain of dystrophin probably adopts a rod shape, as does the middle domain of α -actinin and spec-

trin. The length of a single spectrin triple helical segment has been calculated to be approximately 5 nm (Speicher et al., 1983). Given that the middle domain of dystrophin is formed by 25 triple helical segments, the predicted length of this domain is around 125 nm. A model emphasizing important structural features of the dystrophin molecule is given in Figure 6. The similarity of dystrophin with two distinct proteins that self-assemble as antiparallel dimers (homodimers for α -actinin and heterodimers for spectrin; Wallraff et al., 1986; Speicher et al., 1983) opens the question of the possible dimerization of dystrophin.

Dystrophin was recently demonstrated to be associated with membrane structures (Hoffman et al., 1987c). The primary structure of the protein does not pinpoint the domain of dystrophin that mediates the attachment to membranes. Dystrophin is probably not directly embedded through the membrane, but possibly binds to membranes via an ankyrin-like or protein 4.1-like molecule, in a way similar to the spectrin membrane binding (Bennett and Stenbuck, 1979; Morrow et al., 1980). The C-terminus of dystrophin may be the domain that mediates the attachment to membranes, and it will be interesting to see if this domain shares any similarity with the corresponding domain of β -spectrin, known to contain the ankyrin-binding site (Morrow et al., 1980), or with other cytoskeletal proteins as these protein sequences are entered into the data bases.

The subcellular localization of dystrophin with the transverse tubules of the triadic structure of muscle cells (Hoffman et al., 1987c; Knudson et al., 1988) and the primary structure of the protein presented here suggests that dystrophin is a component of the membrane cytoskeleton. Dystrophin might interact with the myofibrillar actin filaments through the N-terminal actin-binding domain and would then be a link that could maintain the coupling between the triadic structures and the myofibrillar system during muscle contraction (Hoffman et al., 1987c). Alternatively, dystrophin might play a role in the morphogenesis of the T-tubules. Both hypotheses could account for the occurrence of disorganized T-tubule system networks in the muscle of affected patients (Oguchi et al., 1982; Watkins and Cullen, 1987).

Evolution of the Dystrophin Protein

The extensive similarity between dystrophin and α -actinin over three major domains (see Figure 4) strongly suggests that these proteins have evolved from a common ancestor. Both proteins form a new gene family that may include spectrin as well. The sequence of the C-terminus of the β -spectrin is needed to define more precisely the structural and functional relationship between dystrophin and spectrin. The many repeat domains within dystrophin probably arose by multiple steps of domain duplication. It is tempting to speculate that the unit of duplication corresponds to the block of exon whose splice points have been conserved at the beginning and end of a repeat. The unit of duplication does not seem to be the triple helical segment itself, but the last half of a segment plus the first half of the following segment. This unit allows the duplication of the predicted long α -helix 1 plus 3 (see Figure 3) as a sin-

gle block. The low level of similarity between individual repeats hampers the finding of a higher order of repetition in correlation with the various steps of duplication. Details about the steps of duplication may arise when more about the exon structure of the gene is known.

Functional Significance of Internal Protein Deletions in Becker Patients

Deletions of internal domains represented by a truncated protein in some if not most Becker patients should help decipher the role of each domain. It is particularly intriguing that a deletion of the majority of the putative actin-binding domain could result in a very mild Becker phenotype. This raises two questions: First, does the NH2 domain of dystrophin really have an actin-binding activity? Second, if it does, is the actin-binding activity necessary for dystrophin function? Alternatively, the dystrophic phenotype may be modulated by other genetic factors and/or environmental factors, as suggested in Forrest et al. (1987). Phenotypic differences between Becker patients with identical or similar internal domain deletions should help address the question of the influence of such factors in the progression of the muscular dystrophy. In addition, the direct analysis of dystrophin in the muscle of affected patients (Hoffman et al., 1988) would help define the influence exerted by the level of expression and protein stability on the phenotype. The functional significance of each domain can also be tested *in vitro* by the expression of altered sequences of the DMD cDNA in prokaryotic or eukaryotic expression vectors. The combination of both the *in vivo* and *in vitro* approaches will give new insights into the role of dystrophin in the muscle cytoskeletal network.

Experimental Procedures

cDNA Sequencing

The cDNA sequences were determined by the dideoxynucleotide method (Sanger et al., 1977). The sequence of the first 3830 bp of the human DMD cDNA was previously reported (Koenig et al., 1987; Hoffman et al., 1987a; Gross et al., 1987). The sequence of the remaining 10 kb was first determined on one strand by using the various restriction sites and cDNA clone ends indicated on the cDNA map in Koenig et al. (1987). The sequencing was performed on double-stranded plasmid DNA purified on cesium gradient. The universal primers recommended by Stratagene for the Bluescript vectors were used for sequencing. The cDNA sequence was checked on the second strand with the use of specific oligonucleotide primers synthesized by the Howard Hughes Medical Institute Biopolymers Laboratory (Harvard Medical School, Boston) according to the first strand sequence. The primers were spaced by about 200–300 bp such that the second strand sequence was determined across all the restriction sites used in the first strand sequence determination.

Computer Analysis

The nucleotide sequence and predicted amino acid sequence were analyzed by the programs of the University of Wisconsin Genetics Computer Group (UWGCG; Devereux et al., 1984) and BIONET, Intelligenetics. The amino acid sequence was searched for internal repeats by the method of Maizel and Lenk (1981) using COMPARE and DOT-plot of the UWGCG program. Sequences exhibiting similarities were further compared with the alignment method (BESTFIT program) of Smith and Waterman (1981). Secondary structure and hydropathicity were predicted by PEPLOT (UWGCG), using the algorithms of Chou and Fasman (1978) and Kyte and Doolittle (1982), respectively. The PIR (National Biomedical Research Foundation) protein data base and the

GenBank nucleic acids data base were searched with the predicted dystrophin amino acid sequence and the corresponding nucleotide sequence, respectively, by the programs IFIND (BIONET) and WORD-SEARCH (UWGCG). Both programs were adapted from the algorithm of Wilbur and Lipman (1985).

Exon Border Determination

The cloning of the genomic segment of all the exons from the 5' end of the gene to the DXS164 locus has been completed with the exception of three exons: the two exons of the DXS206 locus (Burghes et al., 1987) and the exon contained in the 3.25 kb HindIII genomic fragment at the 5' end of the gene (Koenig et al., 1987). The exon flanking sequences were determined by sequencing the genomic clones with oligonucleotide primers synthesized by the Howard Hughes Medical Institute Biopolymers Laboratory according to the cDNA sequences. The exon boundaries were deduced by comparing the genomic and cDNA sequences and by comparing the genomic sequences with the splice sites consensus sequences (Mount, 1982). The boundaries of one of the two exons of the DXS206 locus has been published (Heilig et al., 1987), and the boundaries of the remaining two exons for which genomic clones were not available have been inferred from the flanking exons. We cannot exclude the possibility that one of these two inferred exons corresponds to two exons. The 21 exons are present in the following HindIII genomic fragments (size in kb; Koenig et al., 1987): exon 1, 3.2; exon 2, 3.25; exon 3, 4.2; exon 4, 8.5; exon 5, 3.1; exon 6, 8.0; exon 7, 4.6; exons 8 and 9, 7.5; exons 10 and 11, 10.5; exon 12, 4.2; exon 13, 6.6; exons 14 and 15, 2.7; exon 16, 6.0; exon 17, 1.7; exon 18, 12; exon 19, 3.0; exon 20, 7.3; exon 21, 11.

Clinical Information of BMD Patients and Deletion Analysis

Patient #8767 was initially diagnosed as DMD due to an early onset of muscle weakness. The fact that he still walks at age 29 and had an uncle diagnosed as BMD who became wheelchair bound at age 49 indicates that he, too, has BMD. The five other BMD patients (JB, B18, #68, #660, and B60) are described in detail in Monaco et al. (1988). Their clinical history is briefly summarized here. JB has been using a wheelchair since age 13; at age 19, he can, with difficulty, maneuver from the chair. JB had an uncle with BMD who died at age 38. B18 is not confined to a wheelchair at age 16, but does require assistance climbing stairs and raising from the floor. #68 has few functional deficits at age 17, although he shows mild proximal muscle weakness. #660 has been confined to a wheelchair since age 16 and is stable at age 21. B60 still walks at age 25 but he has difficulty climbing stairs. The extent of the genomic deletions was analyzed by Southern blot hybridization as detailed in Aldridge et al. (1984) with the DMD cDNA probes (Koenig et al., 1987).

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EXHIBIT 19

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Aims and Scope

The Journal of Gene Medicine is a print and electronic journal which publishes articles on the science of gene transfer and its clinical applications. The journal will consider articles on all aspects of gene therapy including design and production of vectors, research into the mechanisms underlying gene transfer, preclinical studies including animal models, developmental aspects (large-scale production, toxicology) and clinical trials. The editors particularly welcome articles dealing with the methodological aspects of gene transfer *in vivo*, notably in the context of human studies. Articles addressing more fundamental biological issues which could open up avenues for more effective gene transfer are also welcome.

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In this issue

Gene therapy for rheumatoid arthritis: a review

Rheumatoid arthritis (RA) is a severe systemic autoimmune disease in which chronic synovial inflammation results in destruction of the joints. There is no truly effective treatment for RA. Bessis *et al.* review the current status of a gene therapy approach for the treatment of the disease. Potential strategies include down-regulating mediators of inflammation or articular destruction (such as TNF- α or IL-1) and up-regulating anti-inflammatory cytokines (such as IL-4 and IL-10). The authors also review the gene delivery systems that have been used and they consider local versus systemic, and *in vivo* versus *ex vivo* strategies. *Ex vivo* gene transfer has been investigated using synovial cells, fibroblasts, T cells, dendritic cells, and various xenogeneic cells. Clinical trials have started with retroviruses (*ex vivo*) expressing the IL-1 receptor antagonist and have demonstrated the feasibility of the strategy. The best target molecules remain to be determined and extensive pre-clinical studies will need to be performed. (p. 581)

Cell surface protease targeting of a cytotoxic GALV Env?

Kirkham *et al.* investigated the possibility of targeting the cytotoxic activity of a hyperfusogenic Gibbon Ape Leukaemia virus (GALV) envelope glycoprotein therapeutic gene whilst simultaneously enhancing its immune stimulatory properties via local, matrix-metalloprotease (MMP)-mediated release of human GM-CSF. Hyperfusogenic GALV envelopes, whose expression is known to be highly cytotoxic, were fused at

the N-terminus to 'blocking' domains via MMP-sensitive linkers or control linkers (non-cleavable or factor Xa protease-cleavable linkers) and their cytotoxicity was assessed on MMP positive and negative cell lines. Unlike protease targeting in the context of retroviral vectors, protease activation of the cytotoxicity of GALV envelope by cleavage of a fusion-blocking ligand did not appear to be specifically mediated by cell-surface MMPs. Thus, it appears that specificity of cell-cell fusion mediated by GALV envelope cannot be manipulated in the same fashion as virus-cell fusion. (p. 592)

Optimizing retroviral transduction of murine DCs

Gene transfer using retroviral transduction offers the advantage of long-term transgene expression when developing strategies that use dendritic cells (DC) for immunotherapy. The goal of this study was to define optimal conditions for the transduction of murine bone marrow (BM)-derived DCs. Fresnoy *et al.* report here that protamine sulfate and IL-4 allow to increase DC retroviral transduction, whereas polybrene induced DC apoptosis. DCs generated in GM-CSF plus IL-4 presented however a more mature phenotype. These findings have potential implications in experimental gene therapy. (p. 601)

Retina gene therapy in MPS VI cats

Ho *et al.* successfully used an adeno-associated virus (AAV) to treat the retinal manifestations of the lysosomal storage disease, mucopolysaccharidosis VI (MPS VI), in a cat

model. Subretinal injection of a recombinant AAV carrying the wild-type version of the disease-causing arylsulphatase B gene resulted in correction of the disease phenotype in the affected retinal pigment epithelium (RPE) even in MPS VI cats treated late in the disease process. Therapeutic effects persisted as long as 11 months after treatment. These results support the utility of AAV as a vector for the treatment of RPE-specific as well as lysosomal storage diseases. (p. 613)

Cationic liposomes for transduction by envelope-defective retroviruses

In order to investigate the mechanisms of liposome-enhanced retroviral transduction, Porter complexed cationic lipids with retrovirus particles bearing wild-type, chimeric or no envelope proteins. Stable association of cationic liposomes with retrovirus particles enhanced cell binding in proportion to the increase of transduction kinetics. Binding of virus without envelope protein and/or virus receptor was equivalent, indicating that a non-specific interaction precedes receptor recognition. The intrinsic fusogenicity of DOTAP enabled transduction by non-enveloped virus, in some cases at levels approaching that of enveloped virus. Moreover, DC-Chol/DOPE cationic liposomes significantly enhanced inefficient entry of targeting domains-containing chimeric envelopes. These data have important implications for the development of retroviral vector targeting strategies from the perspectives of the specificity of target cell interaction and compensating for chimeric envelope fusion deficiency. (p. 622)

Improved antisense oligonucleotide induced exon skipping in the *mdx* mouse model of muscular dystrophy

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Abstract

Background Duchenne muscular dystrophy (DMD) is a fatal genetic disorder caused by dystrophin gene mutations that preclude synthesis of a functional protein. One potential treatment of the disorder has utilised antisense oligonucleotides (AOs) to induce removal of disease-associated exons during pre-mRNA processing. Induced in-frame mRNA transcripts encode a shorter but functional dystrophin. We have investigated and improved the design of AOs capable of removing exon 23, and thus the disease-causing nonsense mutation, from mRNA in the *mdx* mouse model of DMD.

Methods H-2K^b-bA58 *mdx* cultures were transfected with complexes of Lipofectin and AOs. Exon skipping was detected by RT-PCR and subsequent protein production was demonstrated by Western blotting. AOs were delivered at a range of doses in order to compare relative efficiencies.

Results We describe effective and reproducible exon 23 skipping with several AOs, including one as small as 17 nucleotides. Furthermore, the location of a sensitive exon 23 target site has been refined, whilst minimum effective doses have been estimated *in vitro*. These doses are significantly lower than previously reported and were associated with the synthesis of dystrophin protein *in vitro*.

Conclusions These results demonstrate the increasing feasibility of an AO-based therapy for treatment of DMD. By refining AO design we have been able to reduce the size and the effective dose of the AOs and have dramatically improved the efficiency of the technique. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords antisense oligonucleotides; dystrophin; exon skipping; Duchenne muscular dystrophy

Introduction

Duchenne muscular dystrophy (DMD) is a fatal neuromuscular condition resulting from an absence of dystrophin protein due to either nonsense or frame-shift mutations in the dystrophin gene [1]. In the absence of genetic screening, DMD has an incidence of 1 in 3500 live male births, with 1 in 3 cases resulting from a *de novo* mutation. Dystrophin negative muscle fibres are weaker and undergo repetitive cycles of damage and repair following muscle contraction. Cycles of degeneration and regeneration are eventually

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exhausted and the muscle is gradually replaced by adipose and connective tissue until patients die from respiratory or cardiac failure, usually before the third decade of life [2]. A milder allelic form of the disease, called Becker muscular dystrophy (BMD), is associated with a range of phenotypes ranging from mild to severe (borderline DMD). BMD dystrophin mutations typically give rise to shortened, in-frame transcripts associated with a dystrophin protein of reduced quantity or quality [3].

Gene therapy strategies for the treatment of DMD have so far been met with disappointment. Gene replacement has been hindered by host immune responses to first-generation viral vectors [4], naked plasmid DNA transfer by direct intramuscular injection is inefficient [5], myoblast transfer and stem cell therapy have been respectively fought to overcome poor survival of transplanted cells [6] and very limited cell recruitment [7], homologous gene (atrophia) upregulation may not compensate for the absence of the various isoforms of dystrophin [9], gene repair strategies mediated by chimeric oligonucleotides [10] or short fragment homologous recombination [11] are inefficient to date but offer potential for *ex vivo* correction of host myoblasts; and aminoglycoside therapy [12] is only applicable to a small subset of (nonsense) mutations that cause the disease.

Recent work by our group [13] and others [14] has revealed the potential for an alternative strategy for treating DMD utilising antisense oligonucleotides (AOs) to induce targeted removal of disease-causing exons from pre-mRNA transcripts during splicing. Unlike most other antisense applications which aim to destroy specific RNA targets, such as targeting oncogenes in cancer therapy [15], producing viable dystrophin mRNA capable of being translated into a semi-functional protein is mandatory. For the application of AOs to DMD to be successful, the chemistry of the AOs must be such that targeted degradation of dystrophin mRNA is avoided. Inclusion of a phosphorothioate (PS) backbone retains the anionic charge and increases resistance to nucleases. More importantly, methylation of the 2' oxygen generates an RNA-like molecule that will evade RNase H mediated degradation of the target induced by DNA-RNA hybrids [16].

Inducing successful exon skipping requires delivering 2'-O-methylated PS AOs to the nucleus of muscle cells and their hybridisation to sequence motifs involved in splicing. Blocking these sequences interferes with spliceosome assembly, thereby redirecting the splicing process into excluding the undesirable exon and the flanking introns from the mature mRNA transcript. AO-modified splicing with therapeutic potential has been reported for the dystrophin pre-mRNA [13,14,17-19], for the β -globin transcripts in thalassemia [20], in survival motor neuron 2 (SMN2) pre-mRNA [21], as well as a cryptic splice site mutation in the *cystic fibrosis transmembrane conductance regulator* (CFTR) gene amongst others [22]. As ~15% of point mutations that cause genetic disease affect pre-mRNA splicing, it is probable that the number of diseases/mutations capable of being treated with

this type of therapy will increase as new mutations are identified [23].

An AO-based approach to DMD therapy has a number of advantages over other therapeutic techniques for DMD. In fact, many of the characteristics of the dystrophin gene that make it a challenge to work with may be regarded as positive features for an AO-based treatment. For example, the dystrophin gene located on the short arm of the X chromosome is in excess of 2.4 Mb in size and 79 exons are processed into a 14-kb mRNA transcript that encodes the full-length 427-kDa muscle-specific dystrophin isoform. This mRNA transcript is larger than the packaging size that can be accommodated by most conventional viral vectors. To address this issue, various mini-gene constructs [24,25] and 'gutless' viral vectors have been developed which can tolerate increased payload as well as minimise host response to viral proteins [26]. Despite the continuing problems of immune responses to the viral vector [27,28] and sometimes the introduced and expressed xenotic dystrophin [29], these mini-genes have largely been successful in restoring dystrophin synthesis and providing some phenotypic improvements to transduced muscle fibres. Mini-gene experiments have unequivocally demonstrated the potential for DMD therapy to succeed and have shown that not all of the dystrophin protein is required to restore its expression and localisation to the sarcolemma of muscle fibres. However, there are three major full-length dystrophin products and numerous smaller isoforms arising from both alternative splicing and at least seven different promoters scattered throughout the gene [9]. Replacement of one form of dystrophin or homologous gene upregulation is unlikely to compensate for these other isoforms. By contrast, AO therapy would utilise the existing control elements and could simultaneously target all isoforms affected by the mutation, assuming access of the small AO molecules to the relevant tissues. One limitation of AO therapy is that it cannot completely cure DMD or restore the lost muscle tissue and depleted muscle precursor cells (myoblasts) that are responsible for muscle regeneration. However, if patients could be treated before excessive muscle damage has occurred, it is possible that demands on myoblasts for repair could be reduced.

Most studies of AO-based therapies for DMD have so far been confined to the *mdx* mouse model of the disease, although recently van Daele et al. [14] reported the application of AO-based therapy to primary human DMD muscle cultures. In this current study, we have used conditionally immortalised cultured myotubes derived from a H-2K^b-bA58 *mdx* mouse to demonstrate vast improvements to AO design and the efficiency of induced exon skipping. We evaluated a number of AOs designed to induce specific removal of exon 23 which contains a nonsense mutation in the *mdx* mouse [30]. A range of doses of AOs were delivered with the cationic liposome Lipofectin and the cells were then assessed for the presence of transcripts skipping exon 23 by RT-PCR, and for the production of dystrophin protein

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by Western blotting. We show here that an AO as small as 17 nucleotides can induce strong and consistent exon skipping and subsequent dystrophin protein synthesis. One AO could induce dystrophin protein at a dose as low as 5 nM. This dose is orders of magnitude lower than reported to cause exon skipping in our laboratory [13] and elsewhere [14,17].

Materials and methods

AO design

We have adopted a nomenclature for naming AOs targeted against dystrophin pre-mRNA that provides

information about species specificity, the target site relative to the target exon and the exact annealing position (and thereby the AO size). Each AO name can be divided into two parts, a descriptive component and an exact coordinate position, as summarised in Figure 1A. A more detailed explanation of the nomenclature can be found on the Internet [31]. All AOs were 2'-O-methylphosphorothioate molecules synthesised and HPLC-purified by Geneworks (Adelaide, Australia). The sequence for intron 23 (Genbank accession AF062380) was used to design the sequences, which are shown schematically in Figure 1B. Several of the AOs have been described previously [13,19] and have been renamed here according to the nomenclature.

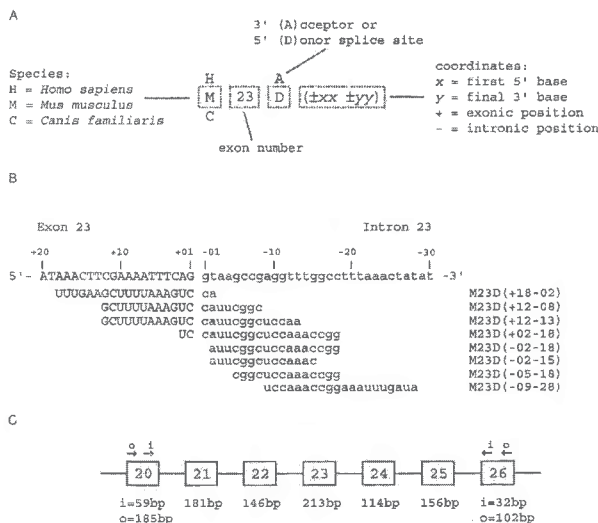


Figure 1. Nomenclature, sequence and location of antisense oligonucleotides and PCR primers. (A) Proposed nomenclature for the naming of AOs targeted against dystrophin pre-mRNA is divided into four parts: species, exon, (D)onor or (A)ceptor splice site and coordinates of the target site relative to the pre-mRNA sequence. (B) Sequences and schematic alignments of the AOs targeted against the donor splice site of intron 23 of mouse dystrophin with details of the numbering employed to assign the AO coordinates. Exonic bases are in upper case and are assigned positive (+) numbers; intronic bases are in lower case and assigned negative (-) numbers. M23D(+12-08) was previously named AO 5'S5-20 [19]; M23D(+12-13) was previously named AO 5'S5-25 [13]. All other AOs have not been previously reported. Sense and scrambled AOs designed as controls for M23D(+02-18) were also utilised and had the following sequences (5' → 3'): AGGUAAGCCGAGUUGGCC and CCUAUGGUCGACAAACGUG, respectively. (C) Relative location of PCR primers and exon sizes for prediction of induced products: exons are represented by boxes and introns by lines (not to scale). Exon sizes in base pairs are indicated. Inner (i) and outer (o) nested PCR primers amplify only a portion of exon 20 or 26, which are also indicated. The sequence of PCR primers has been reported previously [13].

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Cell culture and transfection

H-2K⁰-tsA58 (H-2K) normal and mdx cells were cultured exactly as described previously [12]. Transfections were carried out as follows. Complexes of Lipofectin (Life Technologies, Melbourne, Australia) and AO were always prepared in a 2:1 Lipofectin/AO ratio (w/w) in serum-free Opti-MEM (Life Technologies) according to the manufacturer's instructions, and, unless specified, transfections utilised 1 µg of AO (~300 nM). For the titration experiments (Figure 3), the amount of AO and consequently Lipofectin was varied according to the dose, although the ratio was always maintained at 2:1 (w/w). In all experiments, cells were exposed to AO/Lipofectin complexes for 3 h in serum-free Opti-MEM, after which the media was replaced with DMEM supplemented with 5% horse serum. For the standard transfections and titrations (Figures 2 and 3, respectively), H-2K mdx cells were transfected 48 h after plating in a total volume of 0.5 ml at a density of 2×10^5 cells/well in 24-well plates. Cells were transfected as duplicate wells and extracted RNA was pooled. For RNA time-course experiments (Figure 4), 1×10^5 cells were transfected 24 h after plating in 35-mm dishes. The transfection volume was 2 ml and RNA was extracted from individual dishes every second day after transfection (not plating).

respectively. The same outer and inner (nested) primers spanning exons 20–26 were employed and all reaction components were identical to those reported previously. The relative location of these primers and the sizes of intervening exons are indicated in Figure 1C.

We observed a consistent pattern of bands comprising 'real' products and heteroduplexes when secondary PCR products were electrophoresed through 3% agarose tris/acetate/EDTA (TAE) gels. Heteroduplexes were only observed in samples in which exon skipping had been successfully induced or, less frequently, where naturally occurring revertant fibre transcripts were sporadically amplified. The detection of heteroduplex products is a common problem after PCR amplification of different sized products with complementary regions [32]. To remove the heteroduplexes, we performed S1 nuclease digestion by incubating 10 µl of secondary PCR product with 5 U of S1 Nuclease (Promega, Annandale, Australia) in a 22 µl total reaction volume at 37°C for 18 h. S1-digested products were electrophoresed in 3% TAE agarose to confirm that only the homoduplex products remained. The sizes of PCR products missing one or more exons can be established after gel fractionation but their identities were confirmed by direct sequencing using dye terminator chemistry (ABI; data not shown).

Protein extraction and Western blots

RNA extraction, RT-PCR and S1 nuclease digestion of heteroduplexes

H-2K mdx and H-2K normal cells to be harvested for protein studies were seeded at 1×10^5 cells/well in 6-well plates or 35-mm dishes. For the time-course experiments (Figure 5), H-2K mdx cells were transfected 24 h after plating in 35-mm dishes in a total volume of 2 ml. Protein was extracted from individual dishes after 24 h, then every second day after transfection (not plating). H-2K normal cells were not transfected and protein was harvested every second day after plating to match the time points of the

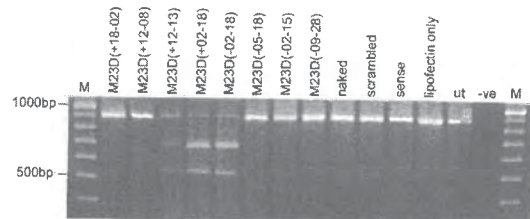


Figure 2. Detection of AO-induced dystrophin mRNAs skipping exon 23 by nested RT-PCR. Cultured H-2K⁰-tsA58 mdx cells were transfected with 1 µg of the respective AO complexed with Lipofectin as described. PCR primers amplified products from within exons 20 and 26. Full-length unspliced (901 bp) mRNA was amplified from all cell extracts but not the PCR negative (-ve) control. Smaller products corresponding to exon 23 (688 bp) and exon 22 and 23 removal (550 bp) were detected in total RNA extracts from cells transfected with M23D(+02-18). M23D(-02-18) and M23D(+12-13). No other AOs or controls were able to induce consistent exon skipping. The gel image represents the PCR products after S1 nuclease digestion to remove heteroduplexes.

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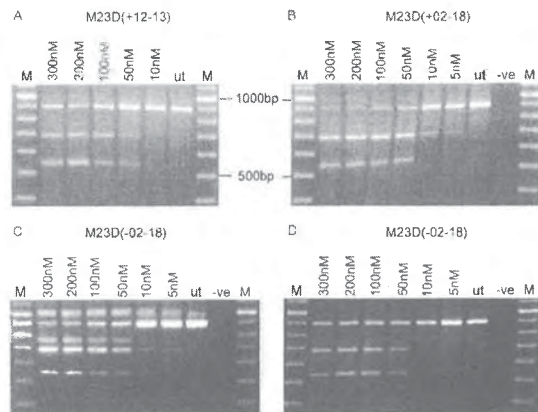


Figure 3. Determination of the minimum effective doses of three antisense oligonucleotides. H-2K^b-tsA58 mdx cells were transfected with M23D(+12-13) (A), M23D(+02-18) (B) and M23D(-02-18) (C and D) at a range of doses to determine the limit at which PCR could amplify products skipping exon 23 (688-bp product). Exon 23 skipping was detected at a dose of 50 nM for M23D(+12-13) and as low as 5 nM for both M23D(+02-18) and M23D(-02-18). (A), (B) and (D) represent R1-PCR products after S1 nuclease digestion to remove heteroduplexes. (C) Typical representation of PCR products before S1 nuclease digestion revealing the complexity of the results if heteroduplexes are not removed.

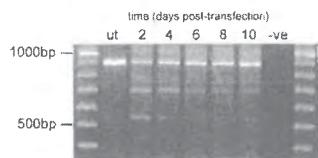


Figure 4. Persistence of induced mRNA transcripts after a single transfection. H-2K^b-tsA58 mdx cells were transfected after 48 h of differentiation in 35-mm dishes with M23D(+02-18) at a dose of 300 nM as described. Total RNA was extracted from a single dish every second day after transfection. Untreated (ut) cells were harvested immediately prior to transfection the remaining cells. mRNA transcripts missing exon 23 were detected for at least 10 days following transfection. Out-of-frame transcripts missing both exon 22 and 23 (350-bp product) were only detectable for 2 days after a single transfection suggesting the possibility that they are removed by nonsense-mediated decay. The image represents the PCR products after S1 nuclease digestion.

transfected H-2K mdx cells. For the dose experiments (Figure 6), cells were transfected 96 h after seeding into 6-well plates in a final transfection volume of 2 ml. Protein

was harvested 96 h after transfection (that is, 8 days after seeding) by first washing the cells twice in phosphate-buffered saline (PBS) and then disrupting the cells in 100 µl of lysis buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 2 M urea and 10% glycerol) containing 3 µl of protease inhibitor cocktail (Sigma, Missouri, USA). Protein extracts were boiled for 5 min and quantitated using a BioRad DC protein assay kit (BioRad, California, USA). Total protein (10 µg per well) was loaded on a pre-cast 4–12% Bis-Tris NuPAGE gel (Invitrogen, Groningen, The Netherlands) run in 1 × MOPS buffer (Invitrogen). Gels were stained with Fast Stain (Zooon Research, Massachusetts, USA) as recommended by the supplier and then scanned with a Hewlett Packard 5370C scanner.

Myosin densitometry was performed on the gel images with the NIH Image Program [33]. Samples were then loaded according to standardised amounts of myosin (~30–50 µg of protein/well) on 5% (37.5:1) polyacrylamide gel containing 0.2% SDS, 375 mM Tris, pH 8.8, and 10% glycerol. Gel-fractionated proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Sydney, Australia) at 1000 mA for 6–7 h in 50 mM Tris, 384 mM glycine and 0.05% SDS. Immediately after transfer, the membrane was washed three times in TBST (10 mM Tris HCl, pH 8.0, 150 mM

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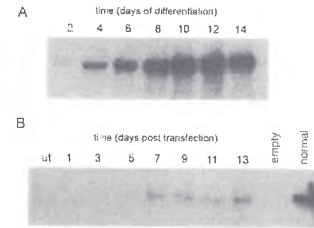


Figure 5. Western blot analysis of dystrophin in untreated H-2K^b-tsA58 normal and transfected H-2K^b-tsA58 mdx cells over time. (A) Protein was extracted from H-2K^b-tsA58 normal cells every second day after plating in 35-mm dishes under differentiation conditions. Protein levels accumulated until day 8 where they reached a maximum. (B) H-2K^b-tsA58 mdx cells were transfected as described in 'Materials and Methods' with 300 nM of M23D(+12-13). Cells were transfected 1 day after seeding into 35-mm dishes under differentiation conditions. Protein was harvested from individual dishes first at 24 h after transfection, then every 2 days so that time points were synchronised with normal cells (A). Dystrophin was undetectable 1 day after transfection (day 2 of differentiation) when levels are also low in normal cells (A). However, low levels of induced dystrophin were detectable after 3 days of transfection and reached a plateau after 7 days (day 8 of differentiation). No dystrophin was detectable in untreated (ut) cells. A protein extract from an 8-day normal sample was loaded as a positive control. All lanes were loaded according to equal amounts of myosin heavy chain.

NaCl, 0.05% Tween 20) and then blocked for 2 h in blocking buffer (5% skim milk powder dissolved in TBST). Incubation with the primary antibody DYS2 (1:100; Novocastra, Newcastle-upon-Tyne, UK) was carried out overnight at 4°C. The membrane was then washed sequentially in TBST, blocking buffer and then washed again in TBST, each for 15 min at room temperature (RT). The primary antibody was detected with a horse radish peroxidase conjugated rabbit anti-mouse antibody (1:500; Dako, California, USA) for 60 min at RT. Prior to

application of the substrate, the membrane was washed twice in TBST and once in TBS each for 10 min. Excess LumLightTM substrate (Roche Molecular Biochemicals, Castle Hill, Australia) was removed after 1 min incubation and the membrane was exposed to Lumifilm (Roche).

Results

Design and testing of new AOs

We have previously reported the successful application of AOs to induce removal of exon 23 from dystrophin mRNA transcripts in cultured mdx cells and muscle *in vivo* [13]. We demonstrated that AOs directed against the donor splice site of intron 23 were able to induce exon 23 skipping while those targeted against the acceptor splice site of intron 23 failed to induce skipping. Of the three AOs that were previously designed to target the 5' splice site, M23D(+12-13), M23D(+12-08)F1TC and M23D(+12-08), we found that M23D(+12-13) was much more efficient at inducing exon 23 removal. This suggested two possibilities, firstly that M23D(+12-13) annealed more efficiently than the other AOs to a sensitive site, or secondly, that AO length plays a decisive role in blocking splice sites. It was our aim to improve the efficiency of exon skipping by better defining susceptible regions and further exploring the relationship between AO size, design and efficiency. Consequently, we evaluated an array of AOs targeted to regions around the donor splice site junction and further into the intron where sensitive or accessible sites lay (Figure 1B). We tested the ability of these newly designed AOs to induce exon 23 skipping by delivering them to cultured H-2K mdx cells as complexes with Lipofectin, as detailed in Materials and Methods.

We first tested M23D(+18-02) and M23D(+02-18), two 20-mer AOs targeted at the exon-intron junction, and designed to have 18 nucleotides complementary to the exon or the intron, respectively, with the remaining two nucleotides hybridising across the junction. M23D(+18-02), which hybridised predominantly to the exon, was unable to induce consistent skipping. By

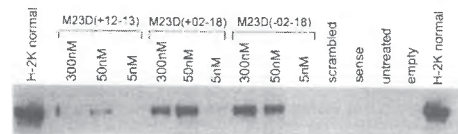


Figure 6. Induction of dystrophin protein at low doses of antisense oligonucleotide. After 4 days of differentiation in 6-well plates, H-2K^b-tsA58 mdx cells were transfected with different doses of AO complexed with Lipofectin. Protein was harvested from all wells simultaneously 4 days after transfection (day 8 of differentiation). M23D(+12-13), M23D(+02-18) and M23D(-02-18) were all able to induce synthesis of a near full-length dystrophin at 300 and 50 nM doses, but only M23D(+02-18) was effective at a dose of 5 nM. No dystrophin was detected in untreated cells, or cells transfected with a 300-nM dose of either the M23D(+02-18) sense and scrambled AOs. A protein extract at day 8 from H-2K^b-tsA58 normal cells was loaded as a time-matched positive control. All lanes were loaded according to equal amounts of myosin heavy chain.

contrast, M23D(+02-18) was very efficient, as judged by the appearance of the 688-bp RT-PCR product missing exon 23 (Figure 2). Additionally, we were also able to detect transcripts skipping both exons 22 and 23 (the 550-bp product) in cells treated with M23D(+02-18). M23D(+18-02) was unable to consistently induce exon 23 skipping even after increasing the dose of the AO (data not shown).

Based on these results, we designed and evaluated several AOs targeted against the intronic region downstream from the 5' splice site of intron 23. Shorter derivatives of M23D(+02-18) were made to economise size requirements for AOs targeted against this region. We also designed AOs to targets located deeper into the intron with the intention of further refining the most susceptible sites. M23D(-02-18), a 17-mer, was able to induce exon skipping, whereas the 14-mers M23D(-05-18) and M23D(-02-15) were unable to induce any detectable skipping (Figure 2). This was despite blocking a smaller but underlapping region to the larger AOs (Figure 1B). Additionally, M23D(-02-18) also induced simultaneous skipping of exons 22 and 23 (the 550-bp product) as was observed with M23D(+02-18) and M23D(+12-13) (Figure 2; Ref. 13) suggesting that the splicing of these two exons is intimately linked. There is no sequence homology between the donor splice sites of introns 22 or 23. Dystrophin mRNA transcripts skipping both exons 22 and 23 are out-of-frame and would not be translated into protein.

Another 20-mer targeted even further into the intron. M23D(-09-28) was also unable to induce exon skipping (Figure 2). A considerable proportion of this AO overlapped the binding site targeted by the successful AOs, i.e., from positions -09 to -13 which does not include any of the consensus splice site sequence. Most interestingly, cells transfected with a combination of 1 µg (~300 nM) each of M23D(+12-08) and M23D(-09-28), both ineffectual AOs in H-2K cells on their own, did not reveal any exon 23 skipping (data not shown). This was despite presumably blocking a 40-nucleotide region encompassing the exon: intron junction and the proposed sensitive region of the intron. Lipofectin only, uncomplexed (naked) AOs, sense and scrambled sequence AOs [designed as controls to M23D(+02-18)] all failed to induce exon 23 skipping (Figure 2). No exon 23 or combined exon 22/23 skipping has ever been observed in untreated or control treated cells. Similarly, transfection of ineffectual AOs at higher doses of 600 nM or 1 µM also failed to induce any exon skipping whilst causing an increase in toxicity (data not shown).

We next sought to compare the three AOs, M23D(+12-13), M23D(+02-18) and M23D(-02-18), that had demonstrated the ability to induce exon skipping, over a range of doses. The AOs M23D(+02-18) and M23D(-02-18) were effective at inducing exon 23 skipping at doses as low as 5 nM in cultured H-2K mdx cells (Figure 3). However, AO M23D(+12-13) required a minimum dose of 50 nM to induce skipping. This difference in AO effectiveness is also apparent in Figure 2 where the

intensity of the 688-bp band representing exon 23 skipping induced by M23D(+12-13) is lower than the intensity of the same product induced by M23D(+02-18) and M23D(-02-18). From this data we concluded that the new AOs had improved exon skipping by ~10-fold over the previously reported AO M23D(+12-13) which had nonetheless induced dystrophin synthesis *in vivo* [13].

Using one of the most efficient AOs, M23D(+02-18), we transfected H-2K mdx cells and extracted RNA every 2 days following transfection. We could routinely detect dystrophin transcripts skipping exon 23 up to 10 days after transfection with a single 300-nM dose (Figure 4). These transcripts were occasionally detected up to 14 days after a single transfection; however, this was not reproducible, because of the limited life span of the cells. Cultures survive poorly beyond 12–14 days and are often highly necrotic at this stage, independent of transfection status. Interestingly, the transcript skipping both exons 22 and 23 was detected for the first 2 days, but rapidly disappeared after this point (Figure 4), whereas the transcript skipping only exon 23 was more persistent. The rapid clearance of the transcript missing exons 22 and 23 was most likely due to nonsense-mediated decay [34] as it is out-of-frame.

We next examined protein extracts from untreated H-2K normal cells and H-2K mdx cells transfected with 300 nM of M23D(+02-18) at various time points. In cultures of normal cells, dystrophin was detectable from the second day after seeding in low-serum differentiation media (Figure 5A). Most H-2K myoblasts have begun fusing into multi-nucleate myotubes by this stage. Dystrophin continued to accumulate, reaching a peak after ~8 days of differentiation, although near maximal levels were achieved after only 6 days in culture. Dystrophin protein appears sooner in H-2K normal cells than has been reported for primary cultures of normal human myoblasts [35] where it was detected in the perinuclear region after day 9, with sarcolemmal localisation not detected until day 14. This discrepancy may be explained by differences between the origins of the cell lines or the greater sensitivity of the Western blot technique versus the immunocytochemistry employed on the primary cultures [35]. Our observations are consistent with previous reports that dystrophin expression increases as mono-nucleate cells mature and fuse to form multinucleate myotubes [36].

We could detect dystrophin protein in AO-treated cultures of H-2K mdx cells, beginning 3 days after transfection, with levels accumulating until day 7 following a single 300-nM dose when protein levels reached a plateau (Figure 5B). These results corroborate the RT-PCR persistence studies (Figure 4) where induced transcripts were present at a low but constant level for several days after a single transfection, thus providing a strong, sustained platform for translation. No size difference between normal and induced dystrophin was expected because the removal of exon 23 deletes only 71 amino acids from a large 427-kDa protein.

Densitometry was performed on the autoradiograph film and estimated that dystrophin levels in AO-treated samples were ~10% of age-matched normal levels by day 12 post-transfection (data not shown). These levels could be regarded as potentially therapeutic compared to untreated control cells since DMD is broadly defined as <3% dystrophin compared to normal [2], and dystrophin levels in transgenic mdx animals around 5% of that in control animals were found to reduce the severity of myopathic symptoms [37]. These levels were the result of only a single transfection and did not require immunoprecipitation or enrichment prior to electrophoresis.

Finally, we compared the ability of the three different effective AOs, M23D(+12-13), M23D(+02-18) and M23D(-02-18), to induce protein after a single transfection across a range of doses. Cells were transfected at day 4 post-seeding as we reasoned dystrophin pre-mRNA expression would be strong at this stage of differentiation, as determined by the accumulating levels of dystrophin protein in H-2K normal cells (Figure 5A). Cells were then harvested 4 days after transfection where dystrophin levels had reached a plateau based on the accumulation studies (Figure 5B). From these titration experiments, M23D(+02-18) was shown to induce a detectable amount of dystrophin at a concentration as low as 5 nM, corresponding to the lowest dose we could detect mRNA transcripts skipping only exon 23 (Figure 3). This was the lowest dose reported so far for inducing either pre-mRNA exon 23 skipping or resultant protein synthesis. Generally, more dystrophin was detected in cultures treated with this AO at the two higher doses tested than in cultures treated with either M23D(+12-13) or M23D(-02-18) at the same doses. We could not detect any dystrophin in extracts of cells treated with a 5-nM dose of M23D(-02-18) or M23D(+12-13). This was unexpected for M23D(-02-18) as we could detect transcripts skipping exon 23 at this dose. However, the lowest effective dose of M23D(+12-13), as judged by the RT-PCR assay, was only 50 nM and no protein was expected at doses lower than this. Of interest was the lower yield of protein induced by this AO compared to M23D(+02-18) and M23D(-02-18), supporting the suggestion that it is less efficient at inducing exon skipping. H-2K mdx cells left untreated or transfected with 300 nM of control AOs did not yield any dystrophin. We employed sense and scrambled sequence AOs [38] both based on M23D(-02-18), the most efficient AO.

Discussion

Developing a gene or genetic therapy for the fatal neuromuscular condition DMD has been an ongoing task since the identification of the dystrophin gene by reverse genetics in the late 1980s [39]. The severity of the condition, its relatively high incidence amongst new born males and the frequency of *de novo* mutations

in probands with no familial history has created an added urgency for developing an effective treatment. To date, most experimental therapies such as virus-mediated gene transfer and cell transplantation have had limited success and been hampered by the complexity and size of the dystrophin gene and the large bulk of muscle to be treated. We, and others, have been developing a new approach for the treatment of DMD utilising AOs to induce targeted exon removal capable of bypassing disease-causing mutations. This approach is still young compared with some of the other potential therapies but we have demonstrated here vast improvements in inducing dystrophin synthesis by refining AO design.

We have designed and tested a number of AOs targeted against the 5' donor splice site of intron 23 in the mdx mouse with the aim of causing specific removal of exon 23, and the mdx nonsense mutation, from the processed dystrophin mRNA. Three of the AOs tested, M23D(+12-13), M23D(+02-18) and M23D(-02-18), successfully induced exon skipping. The efficiency of M23D(+02-18) and M23D(-02-18) at inducing protein synthesis was far superior to that previously reported and represents a substantial improvement in the efficiency of the technique. Detection of mRNA transcripts skipping exon 23 at doses as low as 5 nM with both AOs, and protein at a 5 nM dose with M23D(+02-18), are consequences of improved design. We estimate that the AOs described here are functional at doses 10 fold less than the previously most efficient AO, M23D(+12-13), and 200-fold less than the 1-µM doses reported elsewhere [14,17].

However, comparison of these three AOs to those that failed to induce skipping does not reveal a simple or obvious mode by which they might influence splicing or aid in rational design. A region common to the three successful AOs that lies between and inclusive of nucleotides -09 and -13 of intron 23 (Figure 1B) seems crucial but not absolute for inducing splicing (see below). The results suggest either the presence of a novel splicing motif or a secondary structure within this region that is either sensitive to blocking by AOs, or that mediates hybridisation of AOs. The presence of this region was originally revealed by our previous observation that M23D(+12-08) did not efficiently or reproducibly induce exon 23 skipping in H-2K mdx cells unless an FITC moiety was attached to the 5' end of the AO, the end that overhangs this sensitive region [13]. A number of AOs reported here were designed specifically to refine this region in order to explore and improve AO design.

Two 14-mers, M23D(-02-15) and M23D(-05-18), derived entirely from M23D(+02-18) and M23D(-02-18) both failed to induce exon skipping when targeted against the same sensitive region. Both of these AOs should be able to access the target pre-mRNA if the larger parent AOs are able to do so. However, successful annealing could be dependent on, or initiated by, bases absent from the smaller AOs.

This failure of M23D(-02-15) and M23D(-05-18) is not likely to reflect a minimum effective size for

AOs targeted against splice sites, but a combination of size (*Tm*) and target site sensitivity. Dunckley and co-workers previously reported dystrophin exon skipping with a 14-mer targeted to the acceptor splice site of intron 22 [17], whereas our laboratory has used 14-mers effectively against other exons of the dystrophin pre-mRNA (unpublished observations). This does not rule out the lower annealing temperature (*Tm*) of the smaller 14-mers from contributing to the failed antisense activity at physiological conditions, particularly since the *Tm* of PS AOs is reportedly less than the *Tm* for standard phosphodiester AOs [40]. However, given the relatively low efficiencies and high doses published elsewhere for the smaller AOs, a minimum effective size of ~17 nucleotides is a reasonable threshold to ensure acceptable efficiency and specificity [40] even while smaller AOs may still anneal.

Another possibility for the failure of M23D(-02-15), M23D(-05-18) and M23D(-09-28) is that the target site is more extensive or multifaceted. These AOs block only the putative sensitive region between -09 and -13. It is also significant that blocking the splice site junction alone [with M23D(-18-02), for example] does not cause any consistent exon skipping in H-2K mdx cells. This is despite the fact that the latter region is purportedly essential for both splice site definition and the first of the two transesterification reactions [41]. Assuming the AOs are not inhibited from annealing to their target (see below), the implication is that blocking the intron 23 donor splice site junction does not prevent splice site recognition or intron removal from occurring. This suggests that splicing is either rapid and efficient, occurring before the AO can anneal across the junction, or that splicing occurs irrespective of the presence of the AOs.

However, by blocking the sensitive intronic region as well as the exon: intron junction, it is possible to prevent exon 23 inclusion in the mature mRNA. The three successful AOs target both the region from -09 to -13 and consensus splice site sequences adjacent to the exon: intron junction suggesting that they could derive their effect from blocking both sites. One region, for instance, could preferentially exist in a particular conformation that facilitates strong AO binding, whereas the other could provide the functional moiety necessary for splicing, a moiety that is impeded from functioning by the presence of the AO.

Many of the above conclusions assume that the AOs in question are able to bind to their target. To assess this, we performed bandshift assays [42] as well as RNase H digestion assays (using DNA homologues) [43] of all the AOs described above. We assessed the oligonucleotides at a range of doses with a number of synthetic target RNA fragments of different sizes (data not shown). M23D(-18-02) and M23D(-09-28) were the only AOs able to consistently induce a bandshift under different gel conditions, whereas RNase H digests revealed that all AOs described above were capable of binding to the target at a range of doses and conditions (data not shown). If

these results are to be extrapolated to the cellular environment, it would suggest that even though all our AOs are able to bind to the target pre-mRNA, not all are able to induce skipping. This implies that target site selection is the most crucial aspect of AO design, as only those AOs directed against crucial (active) motifs involved in splicing are able to induce strong and consistent exon skipping. AOs that do not induce skipping are possibly weak competitors of splicing factors, and thus do not induce efficient skipping despite being able to anneal to the target sequence. The synthetic environment of these *in vitro* assays does not, however, reflect the complexity of the myotube cell nucleus where binding may be effected by different salt and ionic conditions as well as the presence of competing proteins (heterogenous nuclear ribonucleoproteins (hnRNPs) or splicing factors themselves, for instance [41]). Additionally, the secondary and tertiary structures of the synthetic RNA are unlikely to reflect the structures of the real dystrophin pre-mRNA as it is transcribed. In a recent paper, van Deutekom and colleagues [14] showed that five out of twelve AOs tested bound to the synthetic dystrophin target and caused a gel mobility shift. However, only four of these AOs were able to induce exon 46 skipping. Our unpublished data from the binding studies suggest strongly that AO functionality cannot be reliably predicted under synthetic conditions, and can only truly be validated by biological assays. Lastly, the various binding assays do not necessarily indicate whether all or part of the AO is involved in binding.

Comparison of normal dystrophin expression to induced mRNA and protein levels in treated H-2K mdx cells suggests a complicated and dynamic scenario. These parameters of AO-based therapy have not been previously reported as they are unique to 'knock-in' approaches where some protein expression is restored, as opposed to more conventional AO applications which seek to down-regulate mRNA transcripts [15]. Understanding these parameters will assist not only AO-based therapy for dystrophin, but potentially AO therapy for other gene products [20,21]. Additionally, dose and treatment regimes will most likely require optimisation for each AO, each exon and each gene target, emphasising the importance of being able to compare and contextualise results of real biological assays with those from other groups or other pre-mRNA targets.

In H-2K normal cells (Figure 5A), dystrophin protein accumulates steadily from the outset of differentiation. The slight lag in the first 2 days is likely due to the low levels of dystrophin mRNA as a consequence of the long 16-h time period necessary to transcribe the 2.4-Mb dystrophin pre-mRNA and co-transcriptionally splice it [44]. This agrees with previous observations that dystrophin transcripts are generally not detectable in undifferentiated (mononucleate) cultures of myogenic cells [36]. H-2K mdx cells are generally fully fused after ~48-h exposure to differentiation conditions. A plateau at the maximum level of expression was reached and maintained from days 8 to 14, the viable limits of the cells in culture.

Improved Dystrophin Exon Skipping

By comparison to normal cells, H-2K mdx cells treated with a single 300 nM dose of M23D(+02-18) at day 1 of differentiation revealed only trace levels of dystrophin protein by 72 h post-transfection, with levels reaching a plateau 7 days after transfection. We estimated these dystrophin levels to be ~10% of normal after a single dose which is within the range capable of relieving myopathic symptoms of DMD [37]. Similarly, induced mRNA transcripts skipping exon 23 were detectable by RT-PCR for up to 10 days following a single transfection, albeit at low levels after 4 days. Thus, the plateau of dystrophin protein observed after day 7 probably reflects a combination of (1) the continuous slow induction of exon 23 removal from nascent dystrophin pre-mRNAs due to the activity of persisting AOs as they are slowly metabolised; (2) the continued translation from persisting induced transcripts; and (3) the stability of translated dystrophin protein. Normal dystrophin protein is reportedly stable in the sarcolemma for 26 weeks *in vivo* [45] and normal mRNA transcripts have an estimated half-life of 16 h [46].

2'-O-Methylation and phosphorothioate backbone modification of AOs is designed to increase their stability and resistance to ubiquitous nucleases, but despite this they are not exempt from eventual degradation. PS AOs are metabolised slower than phosphodiester AOs, principally by a 3' exonuclease activity beginning immediately upon delivery to cells or animals [47,48]. One report suggested that only 10–20% of the full-length 19-labelled PS AOs remained intact in extracts of NIH3T3 cells after 24 h in culture [48]. By assuming similar conditions for H-2K mdx cells, it is likely that only residual amounts of AOs remain after several days of transfection, indicating that induced transcripts skipping exon 23 are relatively stable since, unlike full-length mRNA containing exon 23 they are protected from nonsense-mediated decay [34]. Gradual 3' exonuclease digestion of the AOs could explain why dystrophin protein was not detectable in cell extracts treated with a 5 nM dose of M23D(-02-18) compared to M23D(+02-18). Removal of bases from the 3' end of a shorter AO could decrease the AO's activity faster than metabolism of a larger AO, either by steadily decreasing the *Tm* or by removing regions essential for hybridisation or blocking splice sites. By contrast, removal of 3' bases from M23D(+02-18) would initially make it more similar to M23D(-02-18), an active molecule, before continuing to diminish its activity.

In summary, we have reported vast improvements in the design of AOs able to cause removal of exon 23 from the dystrophin mRNA. The improved AOs were effective at doses substantially lower than previously reported, doses that are theoretically achievable *in vivo*. By comparing successful and failed AOs and the patterns of dystrophin expression and induction, AO design and treatment was shown to require a balance between (1) optimising AO size and stability, and thus the ability to sustain exon skipping even at low levels; (2) transfecting cells expressing reasonable amounts of target pre-mRNA; and (3) allowing sufficient time for protein to translate

and accumulate before assaying, after which the protein should be stable for some time.

Acknowledgements

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RESEARCH ARTICLE

Listeria monocytogenes mediated CFTR transgene transfer to mammalian cells

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Abstract

Background Several approaches for gene therapy of cystic fibrosis using viral and non-viral vectors are currently being undertaken. Nevertheless, the present data suggest that vectors currently being used will either have to be further modified or, alternatively, novel vector systems need to be developed. Recently, bacteria have been proven as suitable vehicles for DNA transfer to a wide variety of eukaryotic cells. In this study, we assessed the ability of the facultative intracellular pathogen *Listeria monocytogenes* to deliver a cDNA encoding the human cystic fibrosis transmembrane conductance regulator (CFTR) to CHO-K1 cells, since these cells have been extensively used for heterologous CFTR expression.

Methods An established in vitro gene transfer system based on antibiotic-mediated lysis of intracellular *L. monocytogenes* was exploited to transfer eukaryotic expression plasmids. Transient as well as stable CFTR transgene expression was analyzed by microscopical and biochemical methods; functionality was tested by whole-cell patch-clamp recordings.

Results *L. monocytogenes* mediated gene transfer to CHO-K1 cells was facilitated by an improved transfection protocol. In addition, the use of the isogenic mutant *L. monocytogenes* hlyW491A, engineered to produce a hemolysin variant with low toxic activity, greatly enhanced the efficiency of gene transfer. This strain allowed the transfer of functional CFTR to CHO-K1 cells.

Conclusions This is the first demonstration of *L. monocytogenes* mediated CFTR transgene transfer. The successful in vitro transfer suggests that *L. monocytogenes* might be a potential vector for cystic fibrosis gene therapy or alternative applications and deserves further investigation in vitro as well as in vivo. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords gene transfer; bacterial vectors; attenuation; CFTR

Introduction

Loss-of-function mutations within the human cystic fibrosis transmembrane conductance regulator (*cftr*) gene are the underlying cause of the frequently occurring inherited disease cystic fibrosis (CF) that often results in lethality early in life [1–3]. Initially, it was found that the *cftr* gene product – the CFTR protein – is a cAMP-regulated chloride channel primarily located in the apical membrane of epithelial cells and central in regulating transcellular electrolyte and fluid transport [4]. However, evidence is accumulating that CFTR has multifaceted properties. Hence, CFTR is involved in the regulation

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EXHIBIT 20

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FILING DATE: *September 23, 2016*

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Certifying Officer

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	AVN-008CN37
		Application Number	
Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		
<p>The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.</p> <p>This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.</p>			

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<input type="checkbox"/>	Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)
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Residence Information (Select One) US Residency <input checked="" type="radio"/> Non US Residency Active US Military Service					
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	AVN-008CN37
		Application Number	
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Title of the Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		
Attorney Docket Number	AVN-008CN37	Small Entity Status Claimed	<input checked="" type="checkbox"/>
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	22	Suggested Figure for Publication (if any)	

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Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	AVN-008CN37
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Prior Application Status	Pending	Remove			
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
	Continuation of	14/740097	2015-06-15		
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Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
14/740097	Continuation of	13/741150	2013-01-14		
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13/741150	Continuation of	13/168857	2011-06-24		
Prior Application Status	Patented	Remove			
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13/168857	Continuation of	12/837359	2010-07-15	8232384B	2012-07-31

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number		AVN-008CN37	
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Title of Invention		ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF			
Prior Application Status		Patented		Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12/837359	Continuation of	11/570691	2008-01-15	7807816B	2010-10-05
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Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
11570691	a 371 of international	PCTAU2005000943	2005-06-28		
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Application Number		Country ⁱ	Filing Date (YYYY-MM-DD)	Access Code ^j (if applicable)	Remove
2004903474		AU	2004-06-28		
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Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

- ☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
- NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	AVN-008CN37
		Application Number	
Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		

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Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		

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Signature	/Amy E. Mandragouras, Esq./	Date (YYYY-MM-DD)	2016-09-23
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	AVN-008CN37
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Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: September 26, 2016
Electronic Signature for Amy E. Mandragouras, Esq.: /Amy E. Mandragouras, Esq./

Docket No.: AVN-008CN37
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Stephen Donald Wilton *et al.*

Application No.: 15/274,772

Confirmation No.: 1042

Filed: September 23, 2016

Art Unit: 1674

For: ANTISENSE OLIGONUCLEOTIDES FOR
INDUCING EXON SKIPPING AND
METHODS OF USE THEREOF

Examiner: Not Yet Assigned

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

FIRST PRELIMINARY AMENDMENT UNDER 37 C.F.R. § 1.115

Dear Sir:

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

Application No.: 15/274,772

Docket No.: AVN-008CN37

AMENDMENTS TO THE CLAIMS

1. **(Cancelled)**

2. **(New)** An antisense oligonucleotide of 25 bases comprising a base sequence that is 100% complementary to 25 consecutive bases of exon 53 of the human dystrophin pre-mRNA, wherein the base sequence comprises at least 17 consecutive bases of C AUU CAA CUG UUG CCU CCG GUU CUG AAG GUG (SEQ ID NO: 193), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.

3. **(New)** A pharmaceutical composition comprising: (i) an antisense oligonucleotide of 25 bases comprising a base sequence that is 100% complementary to 25 consecutive bases of exon 53 of the human dystrophin pre-mRNA, wherein the base sequence comprises at least 17 consecutive bases of C AUU CAA CUG UUG CCU CCG GUU CUG AAG GUG (SEQ ID NO: 193), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping, or a pharmaceutically acceptable salt thereof; and (ii) a pharmaceutically acceptable carrier.

Application No.: 15/274,772

Docket No.: AVN-008CN37

REMARKS

Claim 1 was pending in the application. Claim 1 has been cancelled without disclaimer or prejudice to further prosecution in this or a related application. New claims 2 and 3 have been added. Applicants note that claims 2-3 are identical to claims 21-22, respectively, of U.S. Application No. 14/858,250 (expressly abandoned on January 22, 2016).

Support for new the claims can be found throughout the specification and claims as originally filed. Specifically, support for the term "morpholino antisense oligonucleotide" can be found at page 17, lines 1-5 (Table 1A) of the specification. Morpholino antisense oligonucleotides have been described in the literature. See, *e.g.*, Summerton, J. and Weller, D. (1997) Morpholino Antisense oligomers: design, preparation, and properties. *Antisense Nucl. Acid Drug Dev.*, 7, 187-195; Heasman, J. (2002) Morpholino Oligos: making sense of antisense? *Dev Biol* 243:209-214; and Gebiski, B. *et al.* (2003) Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in *mdx* mouse muscle. *Hum. Mol. Gen.* 12(15): 1801-1811.

No new matter has been added. Accordingly, following entry of the foregoing amendment claims 2 and 3 will be pending in the application.

Application No.: 15/274,772

Docket No.: AVN-008CN37

CONCLUSION

In view of the foregoing, Applicants respectfully submit that the pending claims are in condition for allowance. If a telephone conversation with Applicants' attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 202-4626. If a fee is due with this submission, please charge our Deposit Account No. 12-0080 under Order No. AVN-008CN37, from which the undersigned is authorized to draw.

Dated: September 26, 2016

Respectfully submitted,

Electronic signature: /Amy E. Mandragouras, Esq./
Amy E. Mandragouras, Esq.
Registration No.: 36,207
NELSON MULLINS RILEY & SCARBOROUGH
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Attorney/Agent For Applicant



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/274,772	09/23/2016	Stephen Donald WILTON	AVN-008CN37	1042

123147 7590 09/18/2017
Nelson Mullins Riley & Scarborough LLP/Sarepta
One Post Office Square
Boston, MA 02109

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1674	

NOTIFICATION DATE	DELIVERY MODE
09/18/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipboston.docketing@nelsonmullins.com
chris.schlauch@nelsonmullins.com
ipqualityassuranceboston@nelsonmullins.com

Office Action SummaryApplication No.
15/274,772Applicant(s)
WILTON ET AL.Examiner
KIMBERLY CHONGArt Unit
1674AIA (First Inventor to File)
Status
No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for ReplyA SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 2 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
 - If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
 - Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 06/30/2017.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.
- 2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

- 5) ☒ Claim(s) 2 and 3 is/are pending in the application.
 5a) Of the above claim(s) ____ is/are withdrawn from consideration.
- 6) ☐ Claim(s) ____ is/are allowed.
- 7) ☒ Claim(s) 2 and 3 is/are rejected.
- 8) ☐ Claim(s) ____ is/are objected to.
- 9) ☐ Claim(s) ____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

- 10) ☐ The specification is objected to by the Examiner.
- 11) ☐ The drawing(s) filed on ____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

- a) ☐ All b) ☐ Some** c) ☐ None of the:
1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. ____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☐ Notice of References Cited (PTO-892)
- 2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)
 Paper No(s)/Mail Date 06/30/2017.
- 3) ☐ Interview Summary (PTO-413)
 Paper No(s)/Mail Date. ____
- 4) ☐ Other: ____

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The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Status of Application/Amendment/Claims

Applicant's response filed 06/30/2017 has been considered. Rejections and/or objections not reiterated from the previous office action mailed 12/30/2016 are hereby withdrawn. The following rejections and/or objections are either newly applied or are reiterated and are the only rejections and/or objections presently applied to the instant application. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

With entry of the amendment filed on 06/30/2017, claims 2 and 3 are pending and currently under examination.

Information Disclosure Statement

The submission of the Information Disclosure Statement on 06/30/2017, is in compliance with 37 CFR 1.97. The information disclosure statement has been considered by the examiner and signed copies have been placed in the file.

New Rejections – necessitated by claim amendments

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

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(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 2 and 3 are rejected under pre-AIA 35 U.S.C. 103(a) as being obvious over van Ommen (WO2004/083432 cited on IDS filed 09/26/2016), Koenig et al. (Nature 338, 509 - 511 06 April 1989 cited on IDS filed 09/26/2016) and Bennett et al. (WO 2011/72765 cited on IDS filed 09/26/2016).

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under pre-AIA 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

The claims are drawn to an antisense oligonucleotide of 25 bases comprising a base sequence 100% complementary to consecutive bases of exon 53 of the human dystrophin pre-mRNA, wherein the antisense oligonucleotide base sequence comprises at least 20 consecutive bases of SEQ ID NO: 193, wherein uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, wherein the antisense oligonucleotide is chemically linked to a polyethylene glycol chain

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and wherein the antisense induces exon 53 skipping. The claims are further drawn to a pharmaceutical composition comprising said antisense oligonucleotide.

van Ommen teach a genus of oligonucleotides 16-50 complementary to exon 53 and specifically teach an oligonucleotide h53AON1 that has 18 nucleotides identical to the claimed SEQ ID No. 193 (see Table 2) that causes skipping of exon 53. van Ommen et al. teach the oligonucleotides can be complementary to the exon in the pre-mRNA. Thus given the sequence of the DMD gene has been identified, as demonstrated by Koenig et al., an oligonucleotide sequence complementary to that portion of the mRNA is exactly determined by the simple base pairing rules of DNA and RNA (G being complementary to C, and A being complementary to T (or U)).

vanOmmen et al. the oligonucleotide can have modifications such as morpholino phosphorodiamidate, peptide nucleic acid and locked nucleic acids, for example, and further teach the oligonucleotide comprises modified internucleoside linkages (see claim 12 and page 23). The oligonucleotide taught by van Ommen et al. encompasses both DNA and RNA nucleic acids as well as nucleic acids that are a combination of DNA and RNA as stated on page 9: lines 9-10 "Any oligonucleotide fulfilling the requirements of the invention may be used to induce exon skipping in the DMD gene." van Ommen et al. teach different nucleic acids may be used to generate the oligonucleotide (see page 9 line 30 - page 10). Thus oligonucleotides in which uracil bases are thymine bases are encompassed in the meaning of 'oligonucleotide' taught by van Ommen et al.

van Ommen et al. do not specifically teach the oligonucleotide is chemically linked to a polyethylene glycol chain. Bennett et al. teach oligonucleotides for modifying

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target gene expression and teach oligonucleotides that involve “chemically linking the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to...a polyethylene glycol chain (see page 17, line 33 to page 18, line 26).

It would have been obvious to one of ordinary skill in the art to make an antisense oligonucleotide of 25 bases comprising at least 20 bases of SEQ ID No. 193. Given van Ommen et al. teach a genus of oligonucleotides of up to 50 nucleotides in length, one of skill in the art would have been motivated to extend the sequence of h53AON1 to arrive at oligonucleotides of 25 nucleotides and having 20 nucleotides of SEQ ID No. 193. Because the mRNA sequence containing the exon 53 was known in the prior art, as shown by Keonig et al., the combination of these teachings provides motivation to prepare obvious variants of h53AON1 to try and optimize the activity of the oligonucleotide to prepare the most effective therapeutic for treating DMD.

It would have been routine and a common strategy to try and enhance the oligonucleotide by identifying variants of that oligonucleotide that have a higher level of activity and a common and efficient strategy for doing so is to synthesize and test longer oligonucleotides containing within them the sequence known to have the desired activity. Moreover it would have been obvious and routine to incorporate modifications such as a polyethylene glycol chain to enhance the activity, cellular distribution or cellular uptake of the oligonucleotide as taught by Bennett et al.

Applicant's arguments that there was a high level of unpredictability in the field associated with selecting specific antisense oligonucleotides sequences to induce exon

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skipping will be addressed given it is relevant to the new grounds for rejection. Applicant cites three references and sums the teachings up on page 12 of the response by stating the “references serve to illustrate the unpredictability associated with selecting specific antisense oligonucleotides that are effective for inducing exon skipping of dystrophin exons.”

This argument is not persuasive because van Ommen et al. teach an oligonucleotide that is 18 nucleotides in length. There is no unpredictability in selecting an antisense oligonucleotide to induce exon 53 skipping given van Ommen et al. demonstrates such a compound. Koenig et al. describes the entire DMD cDNA sequence and therefore provides the sequences of exon 53 immediately surround the portion of exon 53 pre-mRNA demonstrated to be sensitive to exon 53 skipping. Thus, one of skill in the art would have a reasonable expectation of success that an oligonucleotide having longer than 18 nucleotides, for example a 25mer comprising at least 20 nucleotides of the claimed SEQ ID No. 193 would induce exon 53 skipping.

Thus in the absence of evidence to the contrary, the invention as a whole would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

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Response to Arguments

Claim Rejections - 35 USC § 102

The rejection of claims 2 and 3 under pre-AIA 35 U.S.C. 102(e) as being anticipated by van Ommen (US Application 20060147952 cited on IDS filed 09/26/2016) is withdrawn in response to claim amendments.

Claim Rejections - 35 USC § 103

The rejection of claims 2 and 3 under pre-AIA 35 U.S.C. 103(a) as being obvious over van Ommen (US Application 20060147952 cited on IDS filed 09/26/2016), van Ommen et al. (Patent 7,973,015 herein after "Patent '0156" cited on IDS filed 09/26/2016), Matteucci, M. (Perspectives in Drug Disc. and Design, 1996, vol. 4, pp 1-16 cited on IDS filed 09/26/2016) and evidence by Koenig et al. (Nature 338, 509 - 511 06 April 1989) is withdrawn in response to claim amendments.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory

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double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to <http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp>.

Claims 2 and 3 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-36 of U.S. Patent No. 8,455,636. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to antisense oligonucleotides having at least 17 consecutive bases of SEQ ID No. 193.

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Claims 2 and 3 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-25 of U.S. Patent No. 8,232,384. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to antisense oligonucleotides having at least 17 consecutive bases of SEQ ID No. 193.

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a).

706.07(a) Final Rejection, When Proper on Second Action [R-07.2015]

Second or any subsequent actions on the merits shall be final, except where the examiner introduces a new ground of rejection that is neither necessitated by applicant's amendment of the claims, nor based on information submitted in an information disclosure statement filed during the period set forth in 37 CFR 1.97(c) with the fee set forth in 37 CFR 1.17(p). Where information is submitted in an information disclosure statement during the period set forth in 37 CFR 1.97(c) with a fee, the examiner may use the information submitted, e.g., a printed publication or evidence of public use, and make the next Office action final whether or not the claims have been amended, provided that no other new ground of rejection which was not necessitated by amendment to the claims is introduced by the examiner. See MPEP § 609.04(b).

Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within

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TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Kimberly Chong whose telephone number is 571-272-3111**. The examiner can normally be reached Monday thru Friday 9-5 pm.

If attempts to reach the examiner by telephone are unsuccessful please contact the SPE for 1674 Ram Shukla at 571-272-0735. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service

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center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see <http://pair-direct.uspto.gov>.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Primary Examiner
Art Unit 1674

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: November 16, 2017
Electronic Signature for Amy E. Mandragouras, Esq.: /Amy E. Mandragouras, Esq./

Docket No.: AVN-008CN37
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Stephen Donald Wilton *et al.*

Application No.: 15/274,772

Confirmation No.: 1042

Filed: September 23, 2016

Art Unit: 1674

For: ANTISENSE OLIGONUCLEOTIDES FOR
INDUCING EXON SKIPPING AND
METHODS OF USE THEREOF

Examiner: K. Chong

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AFTER FINAL ACTION UNDER 37 C.F.R. § 1.116

Dear Sir:

In response to the Final Office Action dated September 18, 2017 (Paper No. 20170911), finally rejecting claims 2 and 3, please amend the above-identified U.S. patent application as follows:

Listing of the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

Application No.: 15/274,772

Docket No.: AVN-008CN37

LISTING OF THE CLAIMS

1. **(Cancelled)**
2. **(Previously Presented)** An antisense oligonucleotide of 25 bases comprising a base sequence that is 100% complementary to 25 consecutive bases of exon 53 of the human dystrophin pre-mRNA, wherein the base sequence comprises at least 20 consecutive bases of C AUU CAA CUG UUG CCU CCG GUU CUG AAG GUG (SEQ ID NO: 193), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, wherein the antisense oligonucleotide induces exon 53 skipping, and wherein the antisense oligonucleotide is chemically linked to a polyethylene glycol chain; or a pharmaceutically acceptable salt thereof.
3. **(Previously Presented)** A pharmaceutical composition comprising: (i) an antisense oligonucleotide of 25 bases comprising a base sequence that is 100% complementary to 25 consecutive bases of exon 53 of the human dystrophin pre-mRNA, wherein the base sequence comprises at least 20 consecutive bases of C AUU CAA CUG UUG CCU CCG GUU CUG AAG GUG (SEQ ID NO: 193), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, wherein the antisense oligonucleotide induces exon 53 skipping, and wherein the antisense oligonucleotide is chemically linked to a polyethylene glycol chain, or a pharmaceutically acceptable salt thereof; and (ii) a pharmaceutically acceptable carrier.

Application No.: 15/274,772

Docket No.: AVN-008CN37

REMARKS

Claims 2 and 3 are pending in the application. Applicants respectfully request reconsideration and withdrawal of the rejections as discussed below. Should the Examiner agree, she is urged to call the undersigned to address any outstanding double patenting rejections to expedite prosecution of this application.

Claim Rejections - 35 U.S.C. § 103(a)

Claims 2 and 3 are rejected under 35 U.S.C. 103(a) as being obvious over van Ommen *et al.* (WO 2004/083432), Koenig *et al.* (Nature 338, 509 - 511 06 April 1989) and Bennett *et al.* (WO 2011/72765). Applicants respectfully traverse this rejection based on the following remarks.

The Office has failed to establish a prima facie case of obviousness

To establish a *prima facie* case of obviousness, the Office must identify both a reason why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed, and why one of ordinary skill in the art would have considered the ***outcome predictable***. *KSR Int'l Co. v. Teleflex, Inc.*, 550 U.S. 398 (2007). Given the deficiencies in the teachings of van Ommen *et al.*, Koenig *et al.* and Bennett *et al.*, there was no motivation to combine the teachings in the manner asserted by the Office. Moreover, given the significant level of unpredictability associated with selecting specific antisense oligonucleotide sequences to induce effective exon skipping, there was no predictability in a successful outcome even if one were to attempt to combine the teachings of the cited references.

The Office has failed to establish it would have been *prima facie* obvious to generate the claimed antisense oligonucleotide with ***all*** the elements provided in the claims. Specifically, the pending claims are drawn to an antisense oligonucleotide having the following elements: (i) 25 bases comprising a base sequence that is 100% complementary to 25 consecutive bases of exon 53 of the human dystrophin pre-mRNA; (ii) 20 consecutive bases of SEQ ID NO: 193; (iii) uracil bases are thymine bases; (iv) the antisense oligonucleotide is a morpholino; (v) the antisense oligonucleotide induces exon 53 skipping; ***and*** (vi) the antisense oligonucleotide is chemically linked to a polyethylene glycol chain.

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The Office relies on van Ommen *et al.* for teaching a genus of antisense oligonucleotides having 16-50 bases complementary to exon 53, and wherein the oligonucleotides can have modifications such as morpholino phosphorodiamidate, peptide nucleic acid and locked nucleic acids. *See* Office Action at page 4. The Office specifically relies on the teaching of an 18-base oligonucleotide (h53AON1), having 18 consecutive nucleotides of SEQ ID NO: 193. *Id.* Koenig *et al.* is relied upon for providing the full sequence of the entire DMD gene. *Id.* The Office asserts that given the sequence of the DMD gene, an oligonucleotide sequence complementary to that portion of the mRNA is exactly determined by the simple base pairing rules of DNA and RNA. *Id.*

The Office admits that van Ommen *et al.* do not specifically teach an oligonucleotide chemically linked to a polyethylene glycol chain, and therefore relies on Bennett *et al.* for teaching this limitation. *Id.* According to the Office, it would have been obvious to one of ordinary skill in the art to lengthen h53AON1 from 18-bases to make an antisense oligonucleotide of 25 bases comprising at least 20 bases of SEQ ID NO: 193 because van Ommen *et al.* teach a genus of oligonucleotides of up to 50 nucleotides in length, and therefore one of skill in the art would have been motivated to extend the sequence of h53AON1 to arrive at the claimed antisense oligonucleotide. *See* Office Action at page 5. The Office asserts the combination of these teachings provides motivation to prepare obvious variants of h53AON1 to try and optimize the activity of the oligonucleotide to prepare the most effective therapeutic for treating DMD. *Id.* Applicants respectfully disagree.

As an initial matter, Applicants wish to point out that there is absolutely nothing in van Ommen *et al.* about substituting uracil bases in RNA oligonucleotides with thymine bases. In fact the word “thymine” (or its structure) is not described anywhere in van Ommen *et al.* The Office suggests that van Ommen *et al.* implicitly disclose the use of thymine bases by arguing that “van Ommen et al. teach different nucleic acids may be used to generate the oligonucleotide...Thus oligonucleotides in which uracil bases are thymine bases are encompassed by the meaning of ‘oligonucleotide’ taught by van Ommen et al.” Office Action at page 4. However, the term “DNA” appears nowhere in van Ommen *et al.* in the context of an antisense oligonucleotide having natural DNA bases. Rather, van Ommen *et al.* states that “[t]his view of the flow of genetic information has prompted the predominantly DNA-based approach for interfering with the protein content of a cell. This view is slowly changing and alternatives for interfering at the DNA level are being pursued,” indicating that the invention

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is an alternative to DNA-based approaches. *See* van Ommen *et al.* at ¶[0003]. Thus, the Examiner's assumption that van Ommen's statement that "[d]ifferent types of nucleic acid may be used to generate the oligonucleotide" (van Ommen *et al.* at ¶[0018]) necessarily is a disclosure of DNA as a nucleic acid that can be used to generate the oligonucleotide, and from this that the use of thymine bases is disclosed, is not supported by anything within the disclosure. Based on these teachings alone, one of skill in the art would not have been motivated to use DNA as presently claimed.

Further, none of the cited references teach or suggest combining the elements to result in the claimed antisense oligonucleotide. Specifically, there is no teaching or suggestion to generate an antisense oligonucleotide of 25 bases, wherein the antisense oligonucleotide comprises 20 consecutive bases of SEQ ID NO: 193, and wherein uracil bases are thymine bases, and wherein the antisense oligonucleotide is a morpholino, and wherein the resulting antisense oligonucleotide induces exon 53 skipping of the human dystrophin pre-mRNA. Therefore, one of ordinary skill in the art would have had no motivation to combine the teachings in the manner suggested by the Officer, and certainly not with a reasonable expectation of success.

Rather, the Office's proposed combination of the teachings of the cited references appears to suggest what may have been "obvious to try" would have been to "vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result." (*In re Kubin*, 561 F.3d 1351, 1359 (Fed. Cir. 2009)). As previously set forth in the Amendment in Response to Non-Final Office Action submitted June 30, 2017 (the "Response"), van Ommen *et al.* describe an empirical approach for the design of exon skipping antisense. *See* Response at page 6. This approach results in non-functional, out-of-frame transcripts, and there is no indication as to what parameters are critical for success. *See Id.* at page 7. Moreover, the results were unpredictable as van Ommen *et al.* report "[t]heir different lengths and G/C contents (%) **did not correlate to their effectivity [sic] in exon skipping**". *Id.* at page 6 (*citing* van Ommen *et al.* Table 2, footnote a [0153]). Koenig *et al.* and Bennett *et al.* fail to provide any teaching or guidance that would make up for the deficiencies in van Ommen *et al.* Koenig *et al.* merely provides the sequence of the DMD gene, and Bennett *et al.* teach oligonucleotides for modifying gene expression.

Accordingly, the cited art provides no indication of what parameters were critical and no direction as to which of many choices is likely to be successful. Moreover, van Ommen *et*

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al. demonstrate the results of testing various antisense oligonucleotides was not predictable. As such, the Office has failed to establish a *prima facie* case of obviousness based on the cited references and Applicants respectfully request reconsideration and withdrawal of this rejection.

High level of unpredictability in the field

With regard to Applicants previously submitted unpredictability arguments, the Office incorrectly asserts that “[t]here is **no unpredictability** in selecting an antisense oligonucleotide to induce exon 53 skipping given van Ommen *et al.* demonstrates [sic] such a compound. Thus, one of skill in the art would have a reasonable expectation of success that an oligonucleotide having longer than 18 nucleotides, for example a 25mer comprising at least 20 nucleotides of the claimed SEQ ID NO: 193 would induce exon 53 skipping.” Office Action at page 6 (emphasis added). Applicants respectfully disagree.

The Office improperly and in contradiction to the Patent Trial and Appeal Board (PTAB) dismissed the objective evidence of unpredictability from those of skill in the art without providing any reason. As provided in the Response, this evidence shows that without sufficient guidance, modifying a starting antisense oligonucleotide is unpredictable. This same evidence was persuasive to the PTAB in Interference 106,007, as described in detail below.

During this proceeding, the critical issue considered by the PTAB was whether selecting an exon 53 skipping antisense oligonucleotide was unpredictable at the time US Application No. 11/233,495, was filed by Academisch Ziekenhuis Leiden (“AZL”). Applicants note the ‘496 application claims priority to the van Ommen *et al.* PCT application presently cited by the Office. Substantial evidence regarding unpredictability at the time of the invention was submitted and considered by the PTAB. Applicants further note Exhibits 2010 and 2015 submitted in the Interference correspond to Aartsma-Rus and Wu *et al.*, previously submitted by Applicants as Appendices A and C, respectively, in the Response filed June 30, 2017.

Upon consideration of this evidence, the PTAB stated “[t]he evidence indicates that at the time AZL filed its application, the identification of AONs that will cause exon skipping was generally thought to be **unpredictable**. One of the significant factors causing that unpredictability is the effect of the number of nucleobases present in the AON.” (Decision on

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Motions at page 17 (emphasis added)). This unpredictability was maintained at the time of the instant invention.

Furthermore, similar to the Office's assertion, AZL argued that upon identification of h53AON1, "one skilled in the art would have investigated extended complementary sequences with the expectation that the longer sequences would bind and cause skipping." *Id.* The PTAB did not find this persuasive at least because AZL failed to provide any publications to support the basis for this expectation. *Id.* at page 18. Like AZL, the Office failed to provide publications to support this expectation. *See* Office Action at page 6. Accordingly, Applicants urge the Office to accept the PTAB's finding of unpredictability in the field of selecting an exon 53 skipping antisense oligonucleotide.

Moreover, the obviousness of changing the length of a base sequence of an antisense oligonucleotide shown to induce exon skipping was also considered by the PTAB. Like the Office Action, the Interference involved, *inter alia*, the 18-base h53AON1 and a 20-base AON sequence. *Id.* at pages 39-40. The PTAB found that "a degree of exon skipping capability would likely be maintained due to a change in a ***small number of complementary nucleobases*** of an AON known to cause skipping" and, therefore, concluded "[i]t would have been obvious, for example, to add the ***two*** complementary nucleobases dictated by the known sequence of exon 53 to either end of h53AON1 with a reasonable expectation that the resultant 20 base AON would cause exon skipping." *Id.* at pages 41-42 (emphasis added). In contrast to the issue considered by the PTAB's and its findings regarding adding ***2 bases*** to lengthen an 18-base sequence to a 20-base sequence, the presently claimed sequence is ***7 bases*** longer than the sequence provided in van Ommen *et al.* Accordingly, it would not have been obvious to extend h53AON1 by 7 bases at least because of the highly degree of unpredictability discussed above, and the Office failed to provide evidence to the contrary.

In summary, the Appendices previously submitted by Applicants along with the Decision of Motions from Interference 106,007, serve to illustrate the unpredictability associated with selecting specific antisense oligonucleotides that are effective for inducing skipping of dystrophin exons. Accordingly, the Office has failed to establish a *prima facie* case of obviousness with respect to the predictability of the outcome in combining teachings of van Ommen *et al.*, Koenig *et al.*, and Bennett *et al.* in the manner proposed to arrive at the claimed invention.

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In view of the preceding remarks, Applicants respectfully request reconsideration and withdrawal of this obviousness rejection.

Double Patenting

Claims 2 and 3 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-36 of U.S. Patent No. 8,455,636. Applicants respectfully request that this rejection be held in abeyance until allowable claims are indicated in the present application.

Claims 2 and 3 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-25 of U.S. Patent No. 8,232,384. Applicants respectfully traverse this rejection.

The Office asserts “the instant claims and the claims of the patent are drawn to antisense oligonucleotides having at least 17 consecutive bases of SEQ ID No. 193.” *Id.* However, Applicants note the instant claims are drawn to antisense oligonucleotide having at least 20 consecutive bases of SEQ ID NO: 193. Moreover, the ‘384 patent is directed to an antisense oligonucleotide *consisting* of SEQ ID NO: 195. ‘384 Patent at claim 1. Applicants point out that there is only a 2 base overlap between SEQ ID NOs: 193 of the ‘384 Patent and SEQ ID NO: 195 of the instant claims. As the present claims are directed to an antisense oligonucleotide having at least 20 consecutive bases of SEQ ID NO: 193, ***11 additional bases*** must be added to SEQ ID NO: 195 to arrive at the instant claims. Here, again, Applicants refer to the high degree of unpredictability discussed above and, accordingly, assert that the claims of the ‘384 patent and the instant claims are patentably distinct from each other. Applicants respectfully request reconsideration and withdrawal of the nonstatutory double-patenting rejection.

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CONCLUSION

If a telephone conversation would expedite prosecution of the application and allowance of the claims, we invite the Examiner to call Applicants' representative at (617) 217-4626. The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 12-0080, under Order No. AVN-008CN37, from which the undersigned is authorized to draw.

Dated: November 16, 2017

Respectfully submitted,
Electronic signature: /Amy E. Mandragouras,
Esq./
Amy E. Mandragouras, Esq.
Registration No.: 36,207
NELSON MULLINS RILEY & SCARBOROUGH LLP
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(617) 217-4626
(617) 217-4699 (Fax)
Attorney/Agent For Applicant

EXHIBIT 21

8145738



THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

August 12, 2021

THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE
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OF:

APPLICATION NUMBER: 14/615,504

FILING DATE: *February 06, 2015*

PATENT NUMBER: 9708361

ISSUE DATE: *July 18, 2017*



Certified by

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	209658-0001-01-US-518587
		Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS		
<p>The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.</p> <p>This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.</p>			

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
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Residence Information (Select One) <input type="radio"/> US Residency <input checked="" type="radio"/> Non US Residency <input type="radio"/> Active US Military Service					
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Inventor 2					Remove
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	Youhei		SATOU		
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Postal Code	305-0003		Country ⁱ	JP	
Inventor 3					Remove
Legal Name					

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	209658-0001-01-US-518587
		Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS		

Prefix	Given Name	Middle Name	Family Name	Suffix
	Shin'ichi		TAKEDA	
Residence Information (Select One) <input type="radio"/> US Residency <input checked="" type="radio"/> Non US Residency <input type="radio"/> Active US Military Service				

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City	Kodaira-shi, Tokyo	State/Province	
Postal Code	187-8551	Country ⁱ	JP

Inventor 4	<div>Remove</div>
Legal Name	

Prefix	Given Name	Middle Name	Family Name	Suffix
	Tetsuya		NAGATA	
Residence Information (Select One) <input type="radio"/> US Residency <input checked="" type="radio"/> Non US Residency <input type="radio"/> Active US Military Service				

City	Kodaira-shi, Tokyo	Country of Residence ⁱ	JP
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Mailing Address of Inventor:

Address 1	c/o National Center of Neurology and Psychiatry		
Address 2	1-1, Ogawahigashicho 4-chome		
City	Kodaira-shi, Tokyo	State/Province	
Postal Code	187-8551	Country ⁱ	JP

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the Add button.		<div>Add</div>
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Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).	
<input type="checkbox"/> An Address is being provided for the correspondence information of this application.	
Customer Number	055694
Email Address	DBRIPDocket@dbr.com <div>Add Email</div> <div>Remove Email</div>

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	209658-0001-01-US-518587
		Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS		

Application Information:

Title of the Invention	ANTISENSE NUCLEIC ACIDS		
Attorney Docket Number	209658-0001-01-US-518587	Small Entity Status Claimed	<input type="checkbox"/>
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	19	Suggested Figure for Publication (if any)	

Filing By Reference :

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

<input type="checkbox"/> Request Early Publication (Fee required at time of Request 37 CFR 1.219)
<input type="checkbox"/> Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.			
Please Select One:	<input checked="" type="radio"/> Customer Number	<input type="radio"/> US Patent Practitioner	<input type="radio"/> Limited Recognition (37 CFR 11.9)
Customer Number	055694		

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	209658-0001-01-US-518587
		Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

When referring to the current application, please leave the application number blank.

Prior Application Status	Pending	<input type="button" value="Remove"/>	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	Continuation of	13891520	2013-04-10
Prior Application Status	Expired	<input type="button" value="Remove"/>	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
13891520	a 371 of international	PCT/JP2011/070318	2011-08-31
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the Add button.			<input type="button" value="Add"/>

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

<input type="button" value="Remove"/>			
Application Number	Country ⁱ	Filing Date (YYYY-MM-DD)	Access Code ^j (if applicable)
2010-196032	JP	2010-09-01	
Additional Foreign Priority Data may be generated within this form by selecting the Add button.			<input type="button" value="Add"/>

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	209658-0001-01-US-518587
		Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS		

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

<input type="checkbox"/>	This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013. NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.
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Authorization to Permit Access:

<input checked="" type="checkbox"/> Authorization to Permit Access to the Instant Application by the Participating Offices
<p>If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.</p> <p>In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.</p> <p>In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing this Authorization.</p>

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	209658-0001-01-US-518587
		Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS		

Applicant 1				Remove	
<p>If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.</p>					
Clear					
<input checked="" type="radio"/> Assignee		<input type="radio"/> Legal Representative under 35 U.S.C. 117		<input type="radio"/> Joint Inventor	
<input type="radio"/> Person to whom the inventor is obligated to assign.			<input type="radio"/> Person who shows sufficient proprietary interest		
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:					
Name of the Deceased or Legally Incapacitated Inventor :					
If the Applicant is an Organization check here. <input checked="" type="checkbox"/>					
Organization Name		NIPPON SHINYAKU CO., LTD.			
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Country ⁱ		JP	Postal Code		601-8550
Phone Number			Fax Number		
Email Address					
Additional Applicant Data may be generated within this form by selecting the Add button.					Add

Applicant 2				Remove	
<p>If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.</p>					
Clear					
<input checked="" type="radio"/> Assignee		<input type="radio"/> Legal Representative under 35 U.S.C. 117		<input type="radio"/> Joint Inventor	
<input type="radio"/> Person to whom the inventor is obligated to assign.			<input type="radio"/> Person who shows sufficient proprietary interest		
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:					
Name of the Deceased or Legally Incapacitated Inventor :					

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	209658-0001-01-US-518587
		Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS		

If the Applicant is an Organization check here. <input checked="" type="checkbox"/>			
Organization Name	NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY		
Mailing Address Information For Applicant:			
Address 1	1-1, Ogawahigashicho 4-chome		
Address 2			
City	Kodaira-shi, Tokyo	State/Province	
Country i	JP	Postal Code	187-8551
Phone Number		Fax Number	
Email Address			
Additional Applicant Data may be generated within this form by selecting the Add button. Add			

Assignee Information including Non-Applicant Assignee Information:

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Assignee 1			
Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.			
Remove			
If the Assignee or Non-Applicant Assignee is an Organization check here. <input checked="" type="checkbox"/>			
Organization Name	NIPPON SHINYAKU CO., LTD.		
Mailing Address Information For Assignee including Non-Applicant Assignee:			
Address 1	14, Kisshoin Nishinosho Monguchicho, Minami-ku		
Address 2			
City	Kyoto-shi, Kyoto	State/Province	
Country i	JP	Postal Code	601-8550
Phone Number		Fax Number	
Email Address			
Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add			

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	209658-0001-01-US-518587
		Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS		

Assignee 2			
Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.			
			<input type="button" value="Remove"/>
If the Assignee or Non-Applicant Assignee is an Organization check here.			<input checked="" type="checkbox"/>
Organization Name	NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY		
Mailing Address Information For Assignee including Non-Applicant Assignee:			
Address 1	1-1, Ogawahigashicho 4-chome		
Address 2			
City	Kodaira-shi, Tokyo	State/Province	
Country i	JP	Postal Code	187-8551
Phone Number		Fax Number	
Email Address			
Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.			<input type="button" value="Add"/>

Signature:

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.					
Signature	/Zhengyu Feng/			Date (YYYY-MM-DD)	2015-02-06
First Name	Zhengyu	Last Name	Feng	Registration Number	66816
Additional Signature may be generated within this form by selecting the Add button.					<input type="button" value="Add"/>

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

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The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/615,504	02/06/2015	Naoki WATANABE	209658-0001-01-US-518587	2704

55694	7590	03/25/2016
DRINKER BIDDLE & REATH (DC)		
1500 K STREET, N.W.		
SUITE 1100		
WASHINGTON, DC 20005-1209		

EXAMINER	
MCGARRY, SEAN	

ART UNIT	PAPER NUMBER
1674	

NOTIFICATION DATE	DELIVERY MODE
03/25/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

DBRIPDocket@dbr.com
 penelope.mongelluzzo@dbr.com

5943

Office Action Summary	Application No. 14/615,504	Applicant(s) WATANABE ET AL.	
	Examiner SEAN MCGARRY	Art Unit 1674	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☐ Responsive to communication(s) filed on _____.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.

3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☒ Claim(s) 1 and 15-25 is/are pending in the application.
5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☒ Claim(s) 1 and 15-25 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10) ☐ The specification is objected to by the Examiner.

11) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a) ☐ All b) ☐ Some** c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. _____.

3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)
Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____.

4) ☐ Other: _____.

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The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 1, 15, and 22-25 are rejected under 35 U.S.C. 101 because the claimed invention is not directed to patent eligible subject matter. Based upon an analysis with respect to the claim as a whole, the claims do not recite something significantly different than a judicial exception. The claimed invention reads on a fragment of a naturally occurring nucleic acid.

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Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

Claims 1-8 and 12-14 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Popplewell et al [US20100168212], Sazani et al [US20100130591] in view of Baker et al [US20130109091] and Bennett et al [US20120190728].

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The claimed invention is drawn to antisense compounds targeted to recited regions all contained within nucleotides 31-61 of exon 53 of the dystrophin gene including antisense oligomers SEQ ID NOS:4, 8, 11, 15, 18, 25, 32, 34, 36, 57, 103, 105, and 109 that cause skipping of the 53rd exon in the human dystrophin gene. The invention includes modifications to the compounds where these modifications are well known and routinely utilized in the antisense art at the time of invention.

Popplewell et al have taught antisense based alteration of splicing in the human dystrophin gene including use as pharmaceuticals. It has been taught to target exon 53 to induce skipping of the 53rd exon. The specific sequences and modifications recited in the instant claims have been clearly suggested by Popplewell et al. See for example SEQ ID NOS:10-12 and 24, and paragraph 15:

The molecule that causes skipping in exon 53 comprises at least a 25 base length from a base sequence selected from:

TABLE-US-00005 (SEQ ID NO: 10) j) CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG; (SEQ ID NO: 11) k) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC; or (SEQ ID NO: 12) l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 53 of the dystrophin gene.

Paragraph 28:

The base "X" in the above base sequences is defined as being thymine (T) or uracil (U). The presence of either base in the sequence will still allow the molecule to bind to the pre-mRNA of the dystrophin gene as it is a complementary sequence. Therefore, the presence of either base in the molecule will cause exon skipping. The base sequence of the molecule may

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contain all thymines, all uracils or a combination of the two. One factor that can determine whether X is T or U is the chemistry used to produce the molecule. For example, if the molecule is a phosphorodiamidate morpholino oligonucleotide (PMO), X will be T as this base is used when producing PMOs. Alternatively, if the molecule is a phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS), X will be U as this base is used when producing 2'OMePSs. Preferably, the base "X" is only thymine (T).

Paragraph 30:

The molecule can be any type of molecule as long as it has the selected base sequence and can bind to a target site of the dystrophin pre-mRNA to cause exon skipping. For example, the molecule can be an oligodeoxyribonucleotide, an oligoribonucleotide, a phosphorodiamidate morpholino oligonucleotide (PMO) or a phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS). Preferably, the oligonucleotide is a PMO. The advantage of a PMO is that it has excellent safety profiles and appears to have longer lasting effects in vivo compared to 2'OMePS oligonucleotides. Preferably, the molecule is isolated so that it is free from other compounds or contaminants.

Paragraph 32:

The molecule is at least 25 bases in length. Preferably, the molecule is at least 28 bases in length. Preferably, the molecule is no more than 35 bases in length and, more preferably, no more than 32 bases in length. Preferably, the molecule is between 25 and 35 bases in length, more preferably, the molecule is between 28 and 32 bases in length, even more preferably, the molecule is between 29 and 31 bases in length, and most preferably, the molecule is 30 bases in length. It has been found that a molecule which is 30 bases in length causes efficient exon skipping. If the molecule is longer than 35 bases in length, the specificity of the binding to the specific exon region is reduced. If the molecule is less than 25 bases in length, the exon skipping efficiency is reduced.

Paragraph 96:

To ensure that the analysis of PMOs for the targeted skipping of exon 53 was not biased by any particular design strategy, seventeen 25mer PMOs were designed to cover the whole of exon 53, with stepwise arrays over suggested bioactive target sites, and then subsequently six 30mer PMOs were designed to target the sequence of exon 53 that showed an association with exon skipping for the 25mers tested. PMOs were designed and tested independently by two different groups (at RHUL and UWA), and then efficacy of the best thirteen sequences confirmed by two other independent groups (at UCL and LUMC). Such a collaborative approach has been used previously as a way of validating target sequences in DMD [4]. Human myoblasts allowed the controlled in vitro comparison of PMO sequences, and confirmation of skipping of

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exon 53 at the RNA level by certain PMOs in both normal cells and, perhaps more importantly, in DMD patient cells with a relevant mutation. These results were further borne out by the expression of dystrophin protein in the DMD cells treated with specific PMOs. Use of the humanised DMD mouse provided an in vivo setting to confirm correct exon exclusion prior to any planned clinical trial. The combined use of these three different systems (normal cells, patient cells and hDMD mouse) as tests of PMO bioactivity provided a reliable and coherent determination of optimal sequence(s) for the targeted skipping of exon 53.

Paragraph 97:

When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated. In normal myoblasts, nucleofection of PMO-G (300 nM) and liposomal-carrier mediated transfection of leashed PMO-G (500 nM) produced over 80% and over 50% skipping of exon 53, respectively, implying that it acts extremely efficiently within the cell. This was confirmed in patient cells. Indeed, this PMO generates the highest levels of exon skipping in patient cells over a range of concentrations (up to 200 nM) and, most important therapeutically, exerts its activity at concentrations as low as 25 nM. The exon skipping activity of this PMO is also persistent, with over 70% exon skipping for 7 days in culture, and over 60% exon skipping for up to three weeks. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMO-G was also shown to skip exon 53 correctly in vivo. These RNA results were further confirmed by the detection of dystrophin protein at a high level in protein extracts from patient cells treated with PMO-G. Previous studies by the Leiden group [18] suggest that the optimal 2'OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured cells and 7% in the hDMD mouse. This 2'OMePS AO shows some degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our optimal PMO is more specific could be a (combined) consequence of the different AO chemistries, length of AO used, and the absolute target site of AO.

The prior art has therefore taught that the same region targeted by the instantly claimed oligomers is superior to other regions of exon 53. The prior art has taught that sequences with SEQ ID NOS:10-12 are included in their invention. The recited SEQ ID NOS: fall squarely within SEQ ID NOS:10-12 and 24 which has been taught by Popplewell to be a “superior” target region of exon 53. While the entire document is pertinent to applicant invention, please also see Example 2 and claims 1-12.

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Sazani et al have also taught antisense oligomers for inducing exon 53 skipping in the human dystrophin gene. Sazani et al have also taught oligomers targeting the same target site and the instant invention and the superior region taught by Popplewell et al See for example SEQ ID NOS: 430, 431, and 628-633 which all overlap or is/are embrace the instantly recited SEQ ID NOS. Sazani et al have also taught that oligomer size choices and modification of antisense oligonucleotides. while the entire reference is relied upon and relevant to applicants invention, applicant is directed to, for example, paragraphs 18-25, 36, 40, 50, 56, 95, 97, 98, 104, 118, 123-177, 196, 197, and claims 36-39, for example.

While the prior art has not specifically disclosed the recited sequences SEQ ID Nos, the prior art has clearly taught that such sequences are embraced within a known target region and furthermore within known antisense compounds. The prior art, however, has taught that the region that the instant compounds are targeted to is a superior target region and furthermore the prior art references taken together have taught that one in the art can alter the sizes of the antisense compounds. It would have been well within the skill of the artisan to test various sizes of oligomers in an optimization of antisense compounds targeting a known superior target region. It is noted that the superior target region is not large; "When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated." Applicants invention is oligomers that are within this exemplified

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compound where it has been clearly taught that sequences within this oligomer were considered by the prior art. The modifications utilized in the invention and recited in the claims were well known and routinely used in the art at the time of invention as shown by the above art and evidenced by Baker et al and Bennett et al. The benefits of the modifications were well known in the art where nuclease protection, and improved hybridization, and cell penetration were known benefits, for example. Both of these references are drawn to antisense compounds utilized in alteration of splicing. See Paragraphs 10, 11, 13, 27, and 60-71 of Baker et al and paragraphs 25, 57-75, 97-104, 140-155, 176, and 180-183 of Bennett et al, for example. Bennett and Baker et al have also taught various size ranges for splice altering antisense compounds.

The invention as a whole would therefore have been *prima facie* obvious to one in the art at the time of invention.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s)

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because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(I)(1) - 706.02(I)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more

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information about eTerminal Disclaimers, refer to

<http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp>.

Claims 1 and 15-25 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 9079934. Although the claims at issue are not identical, they are not patentably distinct from each other because the antisense oligomer of the patent overlaps significantly with and within antisense compounds targeted within or to the regions recited in the instant claim1, for example.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SEAN MCGARRY whose telephone number is (571)272-0761. The examiner can normally be reached on M-Th (7:00-5:30).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Shaojia Anna Jiang can be reached on (571) 272-0627. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Sean R McGarry
Primary Examiner
Art Unit 1674

/SEAN MCGARRY/
Primary Examiner, Art Unit 1674

Docket No.: 209658-0001-01-US-518587
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Naoki WATANABE et al.

Application No.: 14/615,504

Confirmation No.: 2704

Filed: February 6, 2015

Art Unit: 1674

For: ANTISENSE NUCLEIC ACIDS

Examiner: S. McGarry

AMENDMENT / RESPONSE UNDER 37 C.F.R. § 1.111
&
PETITION FOR EXTENSION OF TIME

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Office Action mailed March 25, 2016, Applicants submit the present Amendment / Response. The Office is respectfully requested to consider and enter the following amendments and remarks. Applicants petition herewith for a ONE-month extension of time and submit the corresponding fee, extending the period of response until July 25, 2016.

Amendments to the Abstract begin on page 2.

Amendments to the Claims begin on page 4.

Remarks begin on page 6.

Application No. 14/615,504
Amendment dated July 22, 2016
Reply to Office Action of March 25, 2016

Docket No.: 209658-0001-01-US-518587
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AMENDMENTS TO THE ABSTRACT

Please replace the Abstract with the Substitute Abstract provided on the next page.

Application No. 14/615,504
Amendment dated July 22, 2016
Reply to Office Action of March 25, 2016

Docket No.: 209658-0001-01-US-518587
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SUBSTITUTE ABSTRACT

~~The present invention provides a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.~~

The present invention provides an oligomer which efficiently enables to cause skipping of the 53rd exon in the human dystrophin gene. Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.

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Amendment dated July 22, 2016
Reply to Office Action of March 25, 2016

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AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of the claims in the application:

LISTING OF CLAIMS

Claim 1. (Currently Amended): An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of ~~[[a]]the nucleotide sequence complementary to any one of the sequences consisting of: the 31st to the 55th, the 32nd to the 53rd, the 32nd to the 56th, the 32nd to the 61st, the 33rd to the 54th, the 33rd to the 57th, the 34th to the 58th, the 35th to the 59th, the 36th to the 53rd, the 36th to the 55th, the 36th to the 57th, the 36th to the 60th, or the 37th to the 61st nucleotides from the 5' end of the 53rd exon in the human dystrophin gene of SEQ ID NO: 11 and SEQ ID NO: 57, wherein the antisense oligomer is an oligonucleotide in which the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified, or a morpholino oligomer.~~

Claims 2-16. (Canceled).

Claim 17. (Currently Amended): The antisense oligomer according to claim ~~[[16]]~~1, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH₂, NHR, NR₂, N₃, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

Claim 18. (Currently Amended): The antisense oligomer according to claim ~~[[16]]~~1, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of: a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond, and a boranophosphate bond.

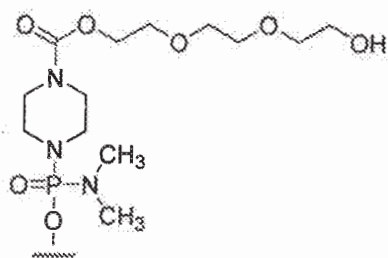
Claim 19. (Previously Presented): The antisense oligomer according to claim 1, which is a morpholino oligomer.

Application No. 14/615,504
Amendment dated July 22, 2016
Reply to Office Action of March 25, 2016

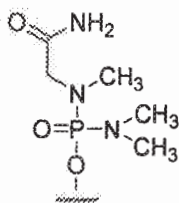
Docket No.: 209658-0001-01-US-518587
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Claim 20. (Previously Presented): The antisense oligomer according to claim 19, which is a phosphorodiamidate morpholino oligomer.

Claim 21. (Previously Presented): The antisense oligomer according to claim 19, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



(2)



(3)

Claim 22. (Canceled).

Claim 23. (Currently Amended): The antisense oligomer according to claim 1, consisting of the nucleotide sequence ~~shown by any one selected from the group consisting of SEQ ID NOS: 4, 8, 11, 15, 18, 25, 32, 34, 36, 57, 103, 104, 105, and 109~~ of SEQ ID NO: 57.

Claim 24. (Currently Amended): The antisense oligomer according to claim 1, consisting of the nucleotide sequence ~~shown by~~ of SEQ ID NO: 11.

Claim 25. (Previously Presented): A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to claim 1, or a pharmaceutically acceptable salt or hydrate thereof.

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REMARKS

Applicants request reconsideration in light of the above amendments and following comments submitted under 37 C.F.R. § 1.111.

1. Amendments to the Abstract

Applicants amend the Abstract to a single paragraph. No prohibited new matter is believed to be introduced.

2. Status of the Claims

The status of the claims following entry of the amendments is as follows:

Claims pending: 1, 17-21, and 23-25

Claims rejected: 1 and 15-25

Claims canceled: 2-16 and 22

Claims amended: 1, 17-18, and 22-23

Applicants amend claim 1 to recite SEQ ID NO: 11 and SEQ ID NO: 57, which respectively correspond to nucleotide sequences complementary to 36th to 60th and 32nd to 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene. Applicants also amend claims 17-18 to update the dependency given the cancellation of claim 16. Applicants further amend claims 22 and 23 to recite SEQ ID NO: 57 and SEQ ID NO: 11, respectively. Thus, no prohibited new matter is believed to be added.

The claims have been amended without prejudice to, or disclaimer of, the canceled subject matter. Applicant reserves the right to file a continuation or divisional application on any subject matter canceled by way of amendments.

3. Information Disclosure Statements

Applicants appreciate the Office's acknowledgement of the Information Disclosure Statements (IDSs) submitted on February 6, 2015, and September 22, 2015.

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4. Status of the Drawings

Applicants respectfully request status as to the acceptance of the drawings as filed February 6, 2015 and Replacement Sheets March 3, 2016 with the Office's next communication.

5. Priority Documents

Applicants respectfully request acknowledgment of the claim for foreign priority and receipt of the priority document(s) with the Office's next communication.

6. Claim Rejection Under 35 U.S.C. § 101

The Office rejects claims 1, 15, and 22-25 as allegedly not directed to patent eligible subject matter. Office Action, page 2. The Office alleges that (1) "the claims do no recite something significantly different than a judicial exception"; and (2) "[t]he claimed invention reads on a fragment of a naturally occurring nucleic acid." *Id.*

Upon entry of the present amendments, claims 15 and 22 stand canceled, mooted at least this aspect of the rejection. Without acquiescing as to the merits of the Office's rejection, amended independent claim 1 recites, *inter alia*, an antisense oligomer that is either (1) a modified oligonucleotide (in which the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide has been modified), or (2) a morpholino oligomer.¹ There is no evidence on the record, or adduced by the Office, that any one of the presently recited antisense oligomers would have existed as "a fragment of a naturally occurring nucleic acid." The Office's rejection is thus moot. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of claims 1 and 23-25.

7. Claim Rejection Under 35 U.S.C. § 103(a)

The Office rejects claims 1 and 15-25² as allegedly obvious over **Popplewell et al.**, US 2010/0168212 ("Popplewell") and **Sazani et al.**, US 2010/0130591 ("Sazani") in view of **Baker**

¹ Morpholino oligomers are synthetic molecules having standard nucleic acid bases bound to morpholine rings (instead of the deoxyribose rings in DNA). See, e.g., Wikipedia page of Morpholino, available at <http://en.wikipedia.org/wiki/Morpholino>.

² The Office alleges "[c]laims 1-8 and 12-14" as be unpatentable over the cited references. Applicants believe that the Office must have meant "claims 1 and 15-25" as indicated in the Office Action Summary (PTOL-326).

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et al., US 2013/0109091 (“Baker”) and Bennett et al., US 2012/0190728 (“Bennett”). Office Action, pages 3-8.

Alleged Grounds for Rejection

Popplewell allegedly teaches targeting the 53rd exon of the human dystrophin gene to induce skipping of the 53rd exon. *Id.*, at 4. Popplewell’s SEQ ID NOs: 10-12 and 24 allegedly suggest the presently recited sequences and modifications. *Id.*

Sazani allegedly teaches antisense oligomers for inducing exon 53 skipping in the human dystrophin gene. *Id.*, at 7. Sazani’s SEQ ID NOs: 430-431 and 628-633 allegedly overlap or encompass the presently recited SEQ ID NOs: 11 or 35. *Id.*

The Office admits that none of the cited references discloses the presently recited SEQ ID NOs. *Id.*, at 8. Nevertheless, the Office alleges that “[i]t would have been well within the skill of the artisan to test various sizes of oligomers in an optimization of antisense compounds targeting a known superior target region.” *Id.*

The Office by relying upon Baker and Bennett further alleges that “[t]he modifications utilized in the invention and recited in the claims were well known and routinely used in the art at the time.” *Id.*, 8. The Office then concludes that “[t]he invention as whole would therefore have been prima facie obvious to one in the art at the time of invention.” *Id.*

Applicants traverse the rejection to the extent it may be applied to the amended claims. Both the suggestion of the claimed invention and the expectation of success must be in the prior art, not in the disclosure of the claimed invention. *In re Dow Chem. Co.*, 837 F.2d 469 (Fed. Cir. 1988). Additionally, once the scope and content of the prior art are determined, the relevant inquiry is whether the prior art suggests all the limitations of the invention, and whether one of ordinary skill in the art would have had a reasonable expectation that the claimed invention would be successful as so combined. *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991).

Upon entry of the present amendments, independent claim 1 recites, *inter alia*, an oligomer consisting of the nucleotide sequence of SEQ ID NO: 11 or SEQ ID NO: 57. As the Office admits, none of the cited references teaches or suggests SEQ ID NO: 11 or SEQ ID NO: 57. The Office’s rejection is unsupported, at least because the Office fails to articulate a rationale why a skilled artisan would have been guided or directed to modify the antisense oligomers of Sazani to arrive at the presently claimed antisense oligomers. Without such

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guidance, the artisan would not have had a reasonable expectation of success in arriving at the claimed sequences. *See, e.g., In re O'Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988).³ Teachings of Baker and Bennett are not directly applicable, because the targeted genes discussed therein differ from the human dystrophin gene.

Additionally, the presently recited oligomers (consisting of the nucleotide sequence of SEQ ID NO: 11 and 57) offer superior skipping effects over the oligomers taught in both Popplewell and Sazani. For example, Figures 2-4 of the Specification (corresponding to data in Test Examples 2-3) show that PMO No. 3 (having the nucleotide sequence of SEQ ID NO: 11; *see* Table 2) outperformed exemplary antisense oligomers taught in Popplewell (PMO Nos. 12 and 15). Additionally, Figures 18-19 of the Specification (corresponding to data in Test Example 7) show that PMO No. 3 (having the nucleotide sequence of SEQ ID NO: 11; *see* Table 2) outperformed exemplary antisense oligomer taught in Sazani (PMO No. 16).⁴ Furthermore, Figures 16-17 (corresponding to data in Test Example 6) show that the oligomer having the nucleotide sequence of SEQ ID NO: 57 (H53_36-60) displays a higher level skipping activity than that having the nucleotide sequence of SEQ ID NO: 11 (H53_32-56). Thus, the recited oligomers consisting of the nucleotide sequence of SEQ ID NO: 57 also have superior skipping activity over exemplary oligomers taught in Popplewell and Sazani. Applicants submit that this superiority is unexpected, at least because none of the cited references teach or suggest such an effect.

Given at least the above arguments, claim 1 as amended and its dependent claims 17-21, and 23-25 would have been nonobvious over cited references. Claims 15-16 and 22 stand canceled, mooted at least this aspect of the rejection. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

³ "The admonition that 'obvious to try' is not the standard under § 103 has been directed mainly at two kinds of error. In some cases, what would have been 'obvious to try' would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful."

⁴ Figure 19 shows that PMO No. 3 has an equivalent level of skipping activity as PMO No. 8. Figure 18 shows that PMO No. 8 has a higher level of skipping activity than the exemplary antisense oligomer taught in Sazani (PMO No. 16). Thus, a skilled artisan given Figures 18-19 would have understood the following order of the skipping activities:

PMO No. 3 ≈ PMO No. 8 >> PMO No. 16.

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8. Double Patenting Rejection

The Office rejects claims 1 and 15-25 on the ground of nonstatutory double patenting as allegedly obvious over claims 1-7 of U.S. Patent No. 9,079,934 (“the ‘934 patent”). Office Action, pages 8-10. The Office alleges:

Although the claims at issue are not identical, they are not patentably distinct from each other because the antisense oligomer of the patent overlaps significantly with and within antisense compounds targeted within or to the regions recited in the instant claim 1, for example.

Id., at 10.

Without acquiescing as to the merits of the Office’s rejection, Applicants amend claim 1 to recite, *inter alia*, an oligomer consisting of the nucleotide sequence of SEQ ID NO: 11 or SEQ ID NO: 57. SEQ ID NO: 11 corresponds to H53_32-56, while SEQ ID NO: 57 corresponds to H53_36-60. The ‘934 patent recites oligomers consisting of the nucleotide sequence of SEQ ID NO: 35, which correspond to H53_36-56. In addition to the sequence differences, the ‘934 patent’s oligomers are shorter (21-mer) than the presently claimed oligomers (25-mer). Applicants respectfully request the Office’s reconsideration given the present claim amendments.

If necessary, Applicants may consider submitting a Terminal Disclaimer Form and payment of the appropriate fee, when the obviousness-type double patenting rejection becomes the only outstanding rejection remaining.

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CONCLUSION

In view of the foregoing, Applicant submits that the pending claims are in condition for allowance, and respectfully request reconsideration and timely allowance of the pending claims. Should the Examiner feel that there are any issues outstanding after consideration of this response; the Examiner is invited to contact Applicant's undersigned representative to expedite prosecution. A favorable action is awaited.

EXCEPT for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. § 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account No. 50-0573. This paragraph is intended to be a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

Dated: July 22, 2016

Respectfully submitted,

Customer Number: 055694

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Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

DBRIPDocket@dbr.com
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5966

Office Action Summary	Application No. 14/615,504		Applicant(s) WATANABE ET AL.	
	Examiner SEAN MCGARRY		Art Unit 1674	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☐ Responsive to communication(s) filed on 7/22/2016.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.

3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☒ Claim(s) 1, 17-21, 24 and 25 is/are pending in the application.
 5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☒ Claim(s) 1, 17-21, 24 and 25 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10) ☐ The specification is objected to by the Examiner.

11) ☒ The drawing(s) filed on 2/6/2015 and 3/3/2015 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12) ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a) ☒ All b) ☐ Some** c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.
2. ☒ Certified copies of the priority documents have been received in Application No. 13/819,520.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)
 Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413)
 Paper No(s)/Mail Date. _____.

4) ☐ Other: _____.

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The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 1, 15, and 22-25 **WERE** rejected under 35 U.S.C. 101 because the claimed invention is not directed to patent eligible subject matter. Based upon an analysis with respect to the claim as a whole, the claims do not recite something significantly different than a judicial exception.

This rejection has been withdrawn in view of applicants amendments to the claims filed 7/22/2016.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which

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said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

Claims 1, 17-21 and 23-25 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Popplewell et al [US20100168212], Sazani et al [US20100130591] in view of Baker et al [US20130109091] and Bennett et al [US20120190728].

The claimed invention is drawn to antisense compounds targeted to recited regions all contained within nucleotides 31-61 of exon 53 of the dystrophin gene including antisense oligomers SEQ ID NOS:4, 8, 11, 15, 18, 25, 32, 34, 36, 57, 103, 105, and 109 that cause skipping of the 53rd exon in the human dystrophin gene. The invention includes modifications to the compounds where these modifications are well known and routinely utilized in the antisense art at the time of invention.

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Popplewell et al have taught antisense based alteration of splicing in the human dystrophin gene including use as pharmaceuticals. It has been taught to target exon 53 to induce skipping of the 53rd exon. The specific sequences and modifications recited in the instant claims have been clearly suggested by Popplewell et al. See for example SEQ ID NOS:10-12 and 24, and paragraph 15:

The molecule that causes skipping in exon 53 comprises at least a 25 base length from a base sequence selected from:

TABLE-US-00005 (SEQ ID NO: 10) j) CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG; (SEQ ID NO: 11) k) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC; or (SEQ ID NO: 12) l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 53 of the dystrophin gene.

Paragraph 28:

The base "X" in the above base sequences is defined as being thymine (T) or uracil (U). The presence of either base in the sequence will still allow the molecule to bind to the pre-mRNA of the dystrophin gene as it is a complementary sequence. Therefore, the presence of either base in the molecule will cause exon skipping. The base sequence of the molecule may contain all thymines, all uracils or a combination of the two. One factor that can determine whether X is T or U is the chemistry used to produce the molecule. For example, if the molecule is a phosphorodiamidate morpholino oligonucleotide (PMO), X will be T as this base is used when producing PMOs. Alternatively, if the molecule is a phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS), X will be U as this base is used when producing 2'OMePSs. Preferably, the base "X" is only thymine (T).

Paragraph 30:

The molecule can be any type of molecule as long as it has the selected base sequence and can bind to a target site of the dystrophin pre-mRNA to cause exon skipping. For example, the molecule can be an oligodeoxyribonucleotide, an oligoribonucleotide, a phosphorodiamidate morpholino oligonucleotide (PMO) or a phosphorothioate-linked 2'-O-methyl oligonucleotide

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(2'OMePS). Preferably, the oligonucleotide is a PMO. The advantage of a PMO is that it has excellent safety profiles and appears to have longer lasting effects in vivo compared to 2'OMePS oligonucleotides. Preferably, the molecule is isolated so that it is free from other compounds or contaminants.

Paragraph 32:

The molecule is at least 25 bases in length. Preferably, the molecule is at least 28 bases in length. Preferably, the molecule is no more than 35 bases in length and, more preferably, no more than 32 bases in length. Preferably, the molecule is between 25 and 35 bases in length, more preferably, the molecule is between 28 and 32 bases in length, even more preferably, the molecule is between 29 and 31 bases in length, and most preferably, the molecule is 30 bases in length. It has been found that a molecule which is 30 bases in length causes efficient exon skipping. If the molecule is longer than 35 bases in length, the specificity of the binding to the specific exon region is reduced. If the molecule is less than 25 bases in length, the exon skipping efficiency is reduced.

Paragraph 96:

To ensure that the analysis of PMOs for the targeted skipping of exon 53 was not biased by any particular design strategy, seventeen 25mer PMOs were designed to cover the whole of exon 53, with stepwise arrays over suggested bioactive target sites, and then subsequently six 30mer PMOs were designed to target the sequence of exon 53 that showed an association with exon skipping for the 25mers tested. PMOs were designed and tested independently by two different groups (at RHUL and UWA), and then efficacy of the best thirteen sequences confirmed by two other independent groups (at UCL and LUMC). Such a collaborative approach has been used previously as a way of validating target sequences in DMD [4]. Human myoblasts allowed the controlled in vitro comparison of PMO sequences, and confirmation of skipping of exon 53 at the RNA level by certain PMOs in both normal cells and, perhaps more importantly, in DMD patient cells with a relevant mutation. These results were further borne out by the expression of dystrophin protein in the DMD cells treated with specific PMOs. Use of the humanised DMD mouse provided an in vivo setting to confirm correct exon exclusion prior to any planned clinical trial. The combined use of these three different systems (normal cells, patient cells and hDMD mouse) as tests of PMO bioactivity provided a reliable and coherent determination of optimal sequence(s) for the targeted skipping of exon 53.

Paragraph 97:

When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated. In normal myoblasts, nucleofection of PMO-G (300 nM) and liposomal-carrier mediated transfection of

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leashed PMO-G (500 nM) produced over 80% and over 50% skipping of exon 53, respectively, implying that it acts extremely efficiently within the cell. This was confirmed in patient cells. Indeed, this PMO generates the highest levels of exon skipping in patient cells over a range of concentrations (up to 200 nM) and, most important therapeutically, exerts its activity at concentrations as low as 25 nM. The exon skipping activity of this PMO is also persistent, with over 70% exon skipping for 7 days in culture, and over 60% exon skipping for up to three weeks. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMO-G was also shown to skip exon 53 correctly in vivo. These RNA results were further confirmed by the detection of dystrophin protein at a high level in protein extracts from patient cells treated with PMO-G. Previous studies by the Leiden group [18] suggest that the optimal 2'OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured cells and 7% in the hDMD mouse. This 2'OMePS AO shows some degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our optimal PMO is more specific could be a (combined) consequence of the different AO chemistries, length of AO used, and the absolute target site of AO.

The prior art has therefore taught that the same region targeted by the instantly claimed oligomers is superior to other regions of exon 53. The prior art has taught that sequences with SEQ ID NOS:10-12 are included in their invention. The recited SEQ ID NOS: fall squarely within SEQ ID NOS:10-12 and 24 which has been taught by Popplewell to be a “superior” target region of exon 53. While the entire document is pertinent to applicant invention, please also see Example 2 and claims 1-12.

Sazani et al have also taught antisense oligomers for inducing exon 53 skipping in the human dystrophin gene. Sazani et al have also taught oligomers targeting the same target site and the instant invention and the superior region taught by Popplewell et al See for example SEQ ID NOS: 430, 431, and 628-633 which all overlap or is/are embrace the instantly recited SEQ ID NOS. Sazani et al have also taught that oligomer size choices and modification of antisense oligonucleotides. while the entire reference is

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relied upon and relevant to applicants invention, applicant is directed to, for example, paragraphs 18-25, 36, 40, 50, 56, 95, 97, 98, 104, 118, 123-177, 196, 197, and claims 36-39, for example.

While the prior art has not specifically disclosed the recited sequences SEQ ID Nos, the prior art has clearly taught that such sequences are embraced within a known target region and furthermore within known antisense compounds. The prior art, however, has taught that the region that the instant compounds are targeted to is a superior target region and furthermore the prior art references taken together have taught that one in the art can alter the sizes of the antisense compounds. It would have been well within the skill of the artisan to test various sizes of oligomers in an optimization of antisense compounds targeting a known superior target region. It is noted that the superior target region is not large; "When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated." Applicants invention is oligomers that are within this exemplified compound where it has been clearly taught that sequences within this oligomer were considered y by the prior art. The modifications utilized in the invention and recited in the claims were well known and routinely used in the art at the time of invention as shown by the above art and evidenced by Baker et al and Bennett et al. The benefits of the modifications were well known in the art where nuclease protection, and improved hybridization, and cell penetration were known benefits, for example. Both of these references are drawn to antisense compounds utilized in alteration of splicing. See

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Paragraphs 10, 11, 13, 27, and 60-71 of Baker et al and paragraphs 25, 57-75, 97-104, 140-155, 176, and 180-183 of Bennett et al, for example. Bennett and Baker et al have also taught various size ranges for splice altering antisense compounds.

The invention as a whole would therefore have been *prima facie* obvious to one in the art at the time of invention.

Response to Arguments

Applicant's arguments filed 7/22/2016 have been fully considered but they are not persuasive.

Applicant has argued that the rejection of record fails to articulate why a skilled artisan would have been guided or directed to modify the antisense oligomers of Sazani to arrive at the presently claimed oligomers. It is noted that the instant oligomers are targeted to nt32-56 and 36-60 of exon 53. These oligomers are 25mers. The examiner asserts that the rejection specifically provides the teaching from the prior art.

From the rejection above:

It has been taught to target exon 53 to induce skipping of the 53rd exon. The specific sequences and modifications recited in the instant claims have been clearly suggested by Popplewell et al. See for example SEQ ID NOS:10-12 and 24, and paragraph 15:

The molecule that causes skipping in exon 53 comprises at least a 25 base length from a base sequence selected from:

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TABLE-US-00005 (SEQ ID NO: 10) j) CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG; (SEQ ID NO: 11) k) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC; or (SEQ ID NO: 12) l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 53 of the dystrophin gene.

Paragraph 32:

The molecule is at least 25 bases in length. Preferably, the molecule is at least 28 bases in length. Preferably, the molecule is no more than 35 bases in length and, more preferably, no more than 32 bases in length. Preferably, the molecule is between 25 and 35 bases in length, more preferably, the molecule is between 28 and 32 bases in length, even more preferably, the molecule is between 29 and 31 bases in length, and most preferably, the molecule is 30 bases in length. It has been found that a molecule which is 30 bases in length causes efficient exon skipping. If the molecule is longer than 35 bases in length, the specificity of the binding to the specific exon region is reduced. If the molecule is less than 25 bases in length, the exon skipping efficiency is reduced.

Paragraph 97:

When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated. In normal myoblasts, nucleofection of PMO-G (300 nM) and liposomal-carrier mediated transfection of leashed PMO-G (500 nM) produced over 80% and over 50% skipping of exon 53, respectively, implying that it acts extremely efficiently within the cell. This was confirmed in patient cells. Indeed, this PMO generates the highest levels of exon skipping in patient cells over a range of concentrations (up to 200 nM) and, most important therapeutically, exerts its activity at concentrations as low as 25 nM. The exon skipping activity of this PMO is also persistent, with over 70% exon skipping for 7 days in culture, and over 60% exon skipping for up to three weeks. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMO-G was also shown to skip exon 53 correctly in vivo. These RNA results were further confirmed by the detection of dystrophin protein at a high level in protein extracts from patient cells treated with PMO-G. Previous studies by the Leiden group [18] suggest that the optimal 2'OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured cells and 7% in the hDMD mouse. This 2'OMePS AO shows some degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our optimal PMO is more specific could be a (combined) consequence of the different AO chemistries, length of AO used, and the absolute target site of AO.

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The prior art has therefore taught that the same region targeted by the instantly claimed oligomers is superior to other regions of exon 53. The prior art has taught that sequences with SEQ ID NOS:10-12 are included in their invention. The recited SEQ ID NOS: fall squarely within SEQ ID NOS:10-12 and 24 which has been taught by Popplewell to be a “superior” target region of exon 53. While the entire document is pertinent to applicant invention, please also see Example 2 and claims 1-12.

Applicant dismissed the Baker and Bennet references since they are not directed to the same gene. This is not what the references are relied on for. For example they are relied on for what was asserted in the rejection above: modifications of antisense compounds utilized for intron splice modulation.

Applicant asserts that the instant compounds have unexpected properties. The examiner disagrees. The compounds function as designed, to alter splicing. The fact that applicant screened for more oligonucleotides in a region that has been taught to be superior utilizing size ranges and modifications known in the art is not unexpected. The prior art indeed asserts that these oligonucleotides are included in their invention as asserted in the rejection of record.

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Double Patenting

Claims 1 and 15-25 **WERE** rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 9079934.

This rejection has been withdrawn in view of the amendments filed 7/22/2016.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SEAN MCGARRY whose telephone number is (571)272-0761. The examiner can normally be reached on M-Th (7:00-5:30).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Shaojia Anna Jiang can be reached on (571) 272-0627. The fax phone

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number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Sean R McGarry
Primary Examiner
Art Unit 1674

/SEAN MCGARRY/
Primary Examiner, Art Unit 1674

Docket No.: 209658-0001-01-US-518587
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Naoki WATANABE et al.

Application No.: 14/615,504

Confirmation No.: 2704

Filed: February 6, 2015

Art Unit: 1674

For: ANTISENSE NUCLEIC ACIDS

Examiner: S. McGarry

AMENDMENT / RESPONSE UNDER 37 C.F.R. § 1.116
&
PETITION FOR EXTENSION OF TIME

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the FINAL Office Action mailed October 27, 2016, the Office is respectfully requested to consider and enter the following amendments and remarks. Applicant petitions herewith a ONE-month extension of time, extending the period of response until February 27, 2017.

Amendments to the Claims begin on page 2.

Remarks begin on page 4.

A Certification and Request for Consideration under the After Final Consideration Pilot Program 2.0 is concurrently submitted.

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Amendment dated February 27, 2017
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AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of the claims in the application:

LISTING OF CLAIMS

Claim 1. (Currently Amended): An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of the nucleotide sequence of ~~SEQ ID NO: 44~~ and SEQ ID NO: 57, wherein the antisense oligomer is an oligonucleotide in which the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified, or a morpholino oligomer.

Claims 2-16. (Canceled).

Claim 17. (Previously Presented): The antisense oligomer according to claim 1, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH₂, NHR, NR₂, N₃, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

Claim 18. (Previously Presented): The antisense oligomer according to claim 1, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of: a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond, and a boranophosphate bond.

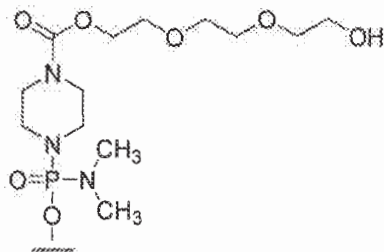
Claim 19. (Previously Presented): The antisense oligomer according to claim 1, which is a morpholino oligomer.

Claim 20. (Previously Presented): The antisense oligomer according to claim 19, which is a phosphorodiamidate morpholino oligomer.

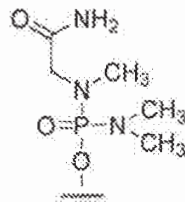
Application No. 14/615,504
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Claim 21. (Previously Presented): The antisense oligomer according to claim 19, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



(2)



(3)

Claims 22-24. (Canceled).

Claim 25. (Previously Presented): A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to claim 1, or a pharmaceutically acceptable salt or hydrate thereof.

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REMARKS

Entry of this Amendment is proper under 37 C.F.R. § 1.116, because the Amendment places the application in condition for allowance for the reasons discussed herein; does not raise any new issue requiring further search and/or consideration, because the amendments amplify issues previously discussed throughout prosecution; relates to matters of form rather than substance, because the added language was already present in the claims and thus presents no additional search burden; adds no new claims; and places the application in a better form for an appeal should an appeal be necessary. The Amendment is necessary and was not earlier presented because it is made in response to arguments raised in the final rejection. Entry of the Amendment, reexamination, and further and favorable reconsideration of the subject application given the following remarks, pursuant to and consistent with 37 C.F.R. § 1.116, are thus respectfully requested.

1. Status of the Claims and Support for the Claim Amendments

The status of the claims following entry of the amendments is as follows:

Claims pending: 1, 17-21, and 25

Claims rejected: 1, 17-21, and 23-25

Claim amended: 1

Claims canceled: 2-16 and 22-24

Applicants amend claim 1 to no longer recite SEQ ID NO: 11. Thus, no prohibited new matter is believed to be added.

The claims have been amended without prejudice to, or disclaimer of, the canceled subject matter. Applicants reserve the right to file a continuation or divisional application on any subject matter canceled by way of amendments.

2. Status the Drawings

Applicants appreciate the Office's acknowledgment that the drawings filed February 6, 2015 and March 3, 2015 are accepted by the Office.

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3. Priority Documents

Applicants appreciate the Office's acknowledgment that certified copies of the priority document have been received in the parent application.

4. Withdrawn Objections and Rejections

Rejections and objections not reiterated stand withdrawn. *See* 37 C.F.R. § 1.113(b); M.P.E.P. §§ 706.07 and 707.07(e).

5. Claim Rejection under 35 U.S.C. § 103(a)

The Office rejects claims 1, 17-21, and 23-25 as allegedly obvious over **Popplewell et al.**, U.S. Published Patent Application No. 2010/0168212 ("Popplewell") and **Sazani et al.**, U.S. Published Patent Application No. 2010/0130591 ("Sazani") in view of **Baker et al.**, U.S. Published Patent Application No. 2013/0109091 ("Baker") and **Bennett et al.**, U.S. Published Patent Application No. 2012/0190728 ("Bennett"). Office Action, pages 2-10.

Alleged Grounds for Rejection

Popplewell allegedly teaches targeting the 53rd exon of the human dystrophin gene to induce skipping of the 53rd exon. *Id.*, at 4. Popplewell's SEQ ID NOs: 10-12 and 24 allegedly suggest the presently recited sequences and modifications. *Id.*, 4 and 8. Sazani allegedly teaches antisense oligomers for inducing exon 53 skipping in the human dystrophin gene. *Id.*, at 6-7. Sazani's SEQ ID NOs: 430-431 and 628-633 allegedly overlap or encompass the presently recited SEQ ID NOs. *Id.* The Office admits that none of the cited references discloses the presently recited SEQ ID NOs. *Id.*, at 8. Nevertheless, the Office alleges that "[i]t would have been well within the skill of the artisan to test various sizes of oligomers in an optimization of antisense compounds targeting a known superior target region." *Id.*

The Office by relying upon Baker and Bennett further alleges that "[t]he modifications utilized in the invention and recited in the claims were well known and routinely used in the art at the time." *Id.*, 7. The Office then concludes that "[t]he invention as whole would therefore have been prima facie obvious to one in the art at the time of invention." *Id.*, 8.

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Given Applicants' Amendment/Response filed July 22, 2016, the Office discounts Applicants' argument. *Id.*, 10. The presently recited SEQ ID Nos "falls squarely within" what "has been taught by Popplewell to be a 'superior' target region." *Id.* The Office further alleges that "[t]he prior art indeed asserts that these oligonucleotides are included in their invention as asserted in the rejection of record." *Id.*

Arguments

Applicants traverse the rejection to the extent it may be applied to the amended claims. Both the suggestion of the claimed invention and the expectation of success must be in the prior art, not in the disclosure of the claimed invention. *In re Dow Chem. Co.*, 837 F.2d 469 (Fed. Cir. 1988). Additionally, once the scope and content of the prior art are determined, the relevant inquiry is whether the prior art suggests all the limitations of the invention, and whether one of ordinary skill in the art would have had a reasonable expectation that the claimed invention would be successful as so combined. *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991).

Upon entry of the present amendments, independent claim 1 recites, *inter alia*, an oligomer consisting of the nucleotide sequence of SEQ ID NO: 57. As the Office admits, none of the cited references teaches or suggests SEQ ID NO: 57. The Office's rejection is unsupported, at least because the Office fails to articulate a rationale why a skilled artisan would have been guided or directed to modify the antisense oligomers of the cited references to arrive at the presently claimed antisense oligomers. Without such guidance, the artisan would not have had a reasonable expectation of success in arriving at the claimed sequences. *See, e.g., In re O'Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988).¹ Teachings of Baker and Bennett are not directly applicable, because the targeted genes discussed therein differ from the human dystrophin gene.

In fact, a skilled artisan given Popplewell would have been directed to use or modify the oligomers that are different from the presently recited ones. Popplewell teaches that the oligomer corresponding to positions 30-59 of exon 53 provides the highest activity. *See, e.g.,*

¹ "The admonition that 'obvious to try' is not the standard under § 103 has been directed mainly at two kinds of error. In some cases, what would have been 'obvious to try' would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful."

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Popplewell, ¶ [0074]² and FIG. 8. In contrast, the presently recited SEQ ID NO: 57 corresponds to positions 36-60 of exon 50. Thus, Popplewell's top performer is different from the presently recited ones. There is no evidence on the record, or adduced by the Office, that a skilled artisan given Popplewell's teachings would have arrived at the presently recited oligomers, let alone doing so with any expectation of success.

Additionally, the presently recited oligomer (consisting of the nucleotide sequence of SEQ ID NO: 57) offer superior skipping effects over the oligomers taught in both Popplewell and Sazani. For example, Figures 2-4 of the Specification (corresponding to data in Test Examples 2-3) show that PMO No. 3 (having the nucleotide sequence of SEQ ID NO: 11; *see* Table 2) outperformed exemplary antisense oligomers taught in Popplewell (PMO Nos. 12 and 15). As shown in TABLE 2, PMO Nos. 12 and 15 corresponds to the top performer taught in Popplewell (targeting sequence 30-59 of exon 53). Additionally, Figures 18-19 of the Specification (corresponding to data in Test Example 7) show that PMO No. 3 (having the nucleotide sequence of SEQ ID NO: 11; *see* Table 2) outperformed exemplary antisense oligomer taught in Sazani (PMO No. 16).³ Furthermore, Figures 16-17 (corresponding to data in Test Example 6) show that the oligomer having the nucleotide sequence of SEQ ID NO: 57 (H53_36-60) displays a higher level skipping activity than that having the nucleotide sequence of SEQ ID NO: 11 (H53_32-56). Thus, the recited oligomers consisting of the nucleotide sequence of SEQ ID NO: 57 also have superior skipping activity over exemplary oligomers taught in Popplewell and Sazani, particularly the top performer taught in Popplewell. Applicants submit that this superiority is unexpected, at least because none of the cited references teach or suggest such an effect.

Given at least the above arguments, claim 1 as amended and its dependent claims 17-21, and 25 would have been nonobvious over cited references. Claims 23-24 stand canceled,

² "...produced the most robust skipping of exon 53, and should be considered the sequence of choice for any upcoming PMO clinical trial."

³ Figure 19 shows that PMO No. 3 has an equivalent level of skipping activity as PMO No. 8. Figure 18 shows that PMO No. 8 has a higher level of skipping activity than the exemplary antisense oligomer taught in Sazani (PMO No. 16). Thus, a skilled artisan given Figures 18-19 would have understood the following order of the skipping activities:

PMO No. 3 ≈ PMO No. 8 >> PMO No. 16.

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mooting at least this aspect of the rejection. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

CONCLUSION

In view of the foregoing, Applicants submit that the pending claims are in condition for allowance, and respectfully request reconsideration and timely allowance of the pending claims. Should the Office have any questions or comments regarding Applicants' amendments or response, please contact Applicants' undersigned representative at (202) 230-5119. Furthermore, please direct all correspondence to the below-listed address.

EXCEPT for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. § 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account No. 50-0573. If an Appeal fee is required to maintain pendency of the present application, the Office is authorized to charge the Appeal fee to the deposit account above and use this paper as a constructive Notice of Appeal.

Dated: February 27, 2017

Respectfully submitted,

Customer Number: 055694

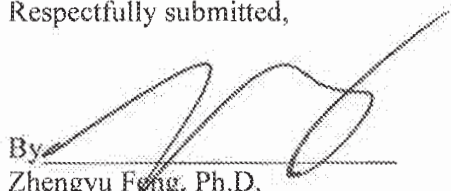
By 
Zhengyu Feng, Ph.D.
Registration No.: 66,816
DRINKER BIDDLE & REATH LLP
1500 K Street, N.W.
Suite 1100
Washington, DC 20005-1209
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Attorneys/Agents For Applicant

EXHIBIT 22

8234739

**THE UNITED STATES OF AMERICA****TO ALL TO WHOM THESE PRESENTS SHALL COME:****UNITED STATES DEPARTMENT OF COMMERCE****United States Patent and Trademark Office**

April 25, 2022

THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE
RECORDS OF THIS OFFICE OF THE FILE WRAPPER AND CONTENTS
OF:

APPLICATION NUMBER: 15/705,172

FILING DATE: September 14, 2017

PATENT NUMBER: 9,994,851

ISSUE DATE: June 12, 2018

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office



W. Montgomery
Wanda Montgomery
Certifying Officer



UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/705,172	09/14/2017	Stephen Donald WILTON	AVN-008CN41	2879

123147 7590 10/05/2017

Nelson Mullins Riley & Scarborough LLP/Sarepta
 One Post Office Square
 Boston, MA 02109

EXAMINER

CHONG, KIMBERLY

ART UNIT

PAPER NUMBER

1674

NOTIFICATION DATE

DELIVERY MODE

10/05/2017

ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipboston.docketing@nelsonmullins.com
 chris.schlauch@nelsonmullins.com
 ipqualityassuranceboston@nelsonmullins.com

Office Action SummaryApplication No.
15/705,172Applicant(s)
WILTON ET AL.Examiner
KIMBERLY CHONGArt Unit
1674AIA (First Inventor to File)
Status
No**-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --****Period for Reply**A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 09/26/2017.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

- 5) ☒ Claim(s) 2 and 3 is/are pending in the application.
 5a) Of the above claim(s) ____ is/are withdrawn from consideration.
- 6) ☐ Claim(s) ____ is/are allowed.
- 7) ☐ Claim(s) ____ is/are rejected.
- 8) ☐ Claim(s) ____ is/are objected to.
- 9) ☒ Claim(s) 2 and 3 are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

- 10) ☐ The specification is objected to by the Examiner.
- 11) ☒ The drawing(s) filed on 09/14/2017 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

- a) ☐ All b) ☐ Some** c) ☐ None of the:
- ☐ Certified copies of the priority documents have been received.
 - ☐ Certified copies of the priority documents have been received in Application No. ____.
 - ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☐ Notice of References Cited (PTO-892)
- 2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)
 Paper No(s)/Mail Date 09/22/2017.
- 3) ☐ Interview Summary (PTO-413)
 Paper No(s)/Mail Date. ____.
- 4) ☐ Other: ____.

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The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Status of Application/Amendment/Claims

Claims 2 and 3 are pending and currently under examination.

Information Disclosure Statement

The submission of the Information Disclosure Statements on 09/22/2017 is in compliance with 37 CFR 1.97. The information disclosure statement has been considered by the examiner and signed copies have been placed in the file.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 2 and 3 are rejected under pre-AIA 35 U.S.C. 103(a) as being obvious over van Ommen (WO2004/083432 cited on IDS filed 09/22/2017) and Koenig et al. (Nature 338, 509 - 511 06 April 1989 cited on IDS filed 09/22/2017).

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The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under pre-AIA 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

The claims are drawn to an antisense oligonucleotide of 20-31 bases comprising a base sequence 100% complementary to consecutive bases of exon 53 of the human dystrophin pre-mRNA, wherein the antisense oligonucleotide base sequence comprises at least 12 consecutive bases of SEQ ID NO: 195, wherein uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense induces exon 53 skipping. The claims are further drawn to a pharmaceutical composition comprising said antisense oligonucleotide.

van Ommen teach a genus of oligonucleotides 16-50 complementary to exon 53 and has identified an active range in the DMD gene and have shown two oligonucleotide h53AON1 and h53AON2 that cause skipping of exon 53 (see Table 2). van Ommen et al. teach the oligonucleotides can be complementary to the exon in the pre-mRNA. Thus given the sequence of the DMD gene has been identified, as demonstrated by Koenig et al., an oligonucleotide sequence complementary to that

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portion of the mRNA is exactly determined by the simple base pairing rules of DNA and RNA (G being complementary to C, and A being complementary to T (or U)).

vanOmmen et al. the oligonucleotide can have modifications such as morpholino phosphorodiamidate, peptide nucleic acid and locked nucleic acids, for example, and further teach the oligonucleotide comprises modified internucleoside linkages (see claim 12 and page 23). The oligonucleotide taught by van Ommen et al. encompasses both DNA and RNA nucleic acids as well as nucleic acids that are a combination of DNA and RNA as stated on page 9: lines 9-10 "Any oligonucleotide fulfilling the requirements of the invention may be used to induce exon skipping in the DMD gene." van Ommen et al. teach different nucleic acids may be used to generate the oligonucleotide (see page 9 line 30 - page 10). Thus oligonucleotides in which uracil bases are thymine bases are encompassed in the meaning of 'oligonucleotide' taught by van Ommen et al.

It would have been obvious to one of ordinary skill in the art to make an antisense oligonucleotide of 20-31 bases comprising at least 12 bases of SEQ ID No. 195. Given van Ommen et al. teach a genus of oligonucleotides of up to 50 nucleotides in length, one of skill in the art would have been motivated to use the sequence of h53AON1 to arrive at oligonucleotides of 20 nucleotides and having 12 nucleotides of SEQ ID No. 195 (which overlaps with 3 nucleotides of h53AON1). Because van Ommen et al. has identified exon 53 and shown oligonucleotides targeting this region can cause exon skipping and because the mRNA sequence containing the exon 53 was known in the prior art, as shown by Keonig et al., the combination of these teachings

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provides motivation to prepare obvious variants of h53AON1 to try and optimize the activity of the oligonucleotide to prepare the most effective therapeutic for treating DMD.

It would have been routine and a common strategy to try and enhance the oligonucleotide by identifying variants of that oligonucleotide that have a higher level of activity and a common and efficient strategy for doing so is to synthesize and test longer oligonucleotides containing within them the sequence known to have the desired activity.

Thus in the absence of evidence to the contrary, the invention as a whole would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP §

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717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to <http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-l.jsp>.

Claims 2 and 3 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-36 of U.S. Patent No. 8,455,636. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to antisense oligonucleotides having at least 17 consecutive bases of SEQ ID No. 193.

Claims 2 and 3 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-25 of U.S. Patent No. 8,232,384. Although the conflicting claims are not identical, they are not patentably

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distinct from each other because the instant claims and the claims of the patent are drawn to antisense oligonucleotides having at least 17 consecutive bases of SEQ ID No. 193.

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a).

706.07(a) Final Rejection, When Proper on Second Action [R-07.2015]

Second or any subsequent actions on the merits shall be final, except where the examiner introduces a new ground of rejection that is neither necessitated by applicant's amendment of the claims, nor based on information submitted in an information disclosure statement filed during the period set forth in 37 CFR 1.97(c) with the fee set forth in 37 CFR 1.17(p). Where information is submitted in an information disclosure statement during the period set forth in 37 CFR 1.97(c) with a fee, the examiner may use the information submitted, e.g., a printed publication or evidence of public use, and make the next Office action final whether or not the claims have been amended, provided that no other new ground of rejection which was not necessitated by amendment to the claims is introduced by the examiner. See MPEP § 609.04(b).

Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any

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extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Kimberly Chong whose telephone number is 571-272-3111**. The examiner can normally be reached Monday thru Friday 9-5 pm.

If attempts to reach the examiner by telephone are unsuccessful please contact the SPE for 1674 Ram Shukla at 571-272-0735. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file

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folder(s) as well as general patent information available to the public. For more information about the PAIR system, see <http://pair-direct.uspto.gov>.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Primary Examiner
Art Unit 1674

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: January 5, 2018

Electronic Signature for Amy E. Mandragouras, Esq.: /Amy E. Mandragouras, Esq./

Docket No.: AVN-008CN41
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Stephen Donald Wilton *et al.*

Application No.: 15/705,172

Confirmation No.: 2879

Filed: September 14, 2017

Art Unit: 1674

For: ANTISENSE OLIGONUCLEOTIDES FOR
INDUCING EXON SKIPPING AND
METHODS OF USE THEREOF

Examiner: K. Chong

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT IN RESPONSE TO NON-FINAL OFFICE ACTION UNDER
37 C.F.R. § 1.111

Dear Sir:

In response to the Office Action dated October 5, 2017 (Paper No. 20171001), please amend the above-identified U.S. patent application as follows:

The **Listing of the Claims** begins on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

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LISTING OF THE CLAIMS

1. **(Canceled)**
2. **(Previously Presented)** An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.
3. **(Previously Presented)** A pharmaceutical composition comprising: (i) an antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping, or a pharmaceutically acceptable salt thereof; and (ii) a pharmaceutically acceptable carrier.

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REMARKS

Claims 2 and 3 are pending in the application. Applicants respectfully request reconsideration and withdrawal of the rejections as discussed below. Should the Examiner agree, she is urged to call the undersigned to address any outstanding double patenting rejections to expedite prosecution of this application.

Claim Rejections - 35 USC § 103

Claims 2 and 3 are rejected under 35 U.S.C. 103(a) as being obvious over van Ommen *et al.* (WO 2004/083432) and Koenig *et al.* (Nature 338, 509 - 511 06 April 1989). Applicants respectfully traverse this rejection based on the following remarks.

The Office failed to establish a prima facie case of obviousness

The Office bears the initial burden of factually supporting any *prima facie* conclusion of obviousness. (MPEP §2142, 9th Ed.) “The Federal Circuit has stated that ‘rejections on obviousness cannot be sustained with mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness.’” (*Id.* citing *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006); see also *KSR*, 550 U.S. at 418, 82 USPQ2d at 1396 (quoting Federal Circuit statement with approval).)

“Obviousness is a question of law with underlying factual findings, including: (1) the scope and content of the prior art; (2) the level of ordinary skill in the pertinent art; (3) the differences between the claimed invention and the prior art; and (4) objective evidence such as commercial success, long-felt need, and the failure of others.” (*KSR Int'l Co. V. Teleflex, Inc.*, 550 U.S. 398 (2007) citing *Graham v. John Deere Co.*, 383 U.S. 1, 17-18 (1966).) With respect to the third inquiry, to establish a *prima facie* case of obviousness, the Office must identify both a reason why a person of ordinary skill in the art would have combined the prior art elements to arrive at the claimed subject matter, and a reason why one of ordinary skill in the art would have considered the outcome predictable. (*KSR Int'l Co. V. Teleflex, Inc.*, 550 U.S. 398 (2007).)

“In cases involving the patentability of a new chemical compound, *prima facie* obviousness under the third *Graham* factor generally turns on the structural similarities and differences between the claimed compound and the prior art compounds.” According to

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established Federal Circuit precedent, a two-part "lead compound" analysis must be satisfied to establish a *prima facie* case of obviousness. (*Otsuka Pharmaceutical Co. Ltd., v. Sandoz, Inc.*, 678 F.3d 1280 (2012).) To satisfy the lead compound analysis, the Office must establish: (1) that one of ordinary skill in the art would have selected the asserted prior art compound as a lead compound for further development, and (2) that the prior art would have motivated one of ordinary skill in the art to modify the lead compound to make the claimed compound with a reasonable expectation of success. (*Id.* at 1291-1292.)

For the reasons below, neither prong of the two part inquiry has been met in the present case. The first prong is not met because the Office failed to provide a reason why one of ordinary skill in the art would have selected SEQ ID NO: 29 ("h53AON1") of van Ommen et al. as a lead compound. The second prong is not met because, even assuming that one of skill in the art would have selected h53AON1 as a lead compound, the Office failed to provide a reason or motivation to specifically **lengthen** h53AON1 by **nine** additional bases of SEQ ID NO: 195 to arrive at the limitation of claim 1 that the base sequence comprises at least 12 consecutive bases of SEQ ID NO: 195.¹ Moreover, there was a significant level of unpredictability associated with selecting a specific antisense oligonucleotide to induce effective exon skipping of human dystrophin pre-mRNA at the time of the invention, and therefore no reasonable expectation of success.

Lead Compound Analysis

i. **The Office failed to provide a reason why a person of ordinary skill in the art would have selected h53AON1 as a lead compound**

A lead compound is "a compound in the prior art that would be most promising to modify in order to improve upon its... activity and obtain a compound with better activity." (*Otsuka Pharmaceutical Co. Ltd., v. Sandoz, Inc.*, at 1291 (citing *Takeda Chemical Industries, Ltd. v. Alphapharm Pty., Ltd.*, 492 F.3d 1350, 1357 (Fed. Cir. 2007)).) "[A] reason to select a compound as a lead compound depends on **more than just structural similarity**..." *Bristol-Myers Squibb Co. v. Teva Pharmaceuticals USA, Inc.*, 923 F.Supp.2d 602 at 657 (2013) (citing *Matrix Labs.*, 619 F.3d at 1354; emphasis added). Notably, it has been held that "absent

¹ Applicants note and further explain below that, contrary to the position of the Office, the skilled artisan must lengthen h53AON1 by nine nucleotides, not two nucleotides, of SEQ ID NO: 195 to achieve the requirement of at least 12 bases of SEQ ID NO: 195 recited by the instant claims.

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a reason or motivation based on such prior art evidence, *mere structural similarity* between a prior art compound and the claimed compound *does not inform the lead compound selection.*" (*Otsuka Pharmaceutical Co. Ltd., v. Sandoz, Inc.*, at 1292 (citing *Daiichi Sankyo Co. v. Matrix Labs., Ltd.*, 619 F.3d 1346, 1354 (Fed. Cir. 2010)); emphasis added.)

The Office has not provided any evidence or reasoning to support the conclusion that a person of ordinary skill in the art would have selected h53AON1 as the lead compound. Instead, the Office simply chooses it as its basis for the alleged obviousness of the claimed subject matter. Thus, its' selection by the Office in the absence of any supporting evidence or reasoning as a lead compound can only be through impermissible hindsight. Accordingly, the Office has not established that a person of ordinary skill in the art would select h53AON1 as the lead compound to modify to arrive at the claimed antisense oligonucleotides. For this reason alone, the claims are not *prima facie* obvious over the cited documents, and the Office should therefore withdraw the rejection.

ii. *The cited art does not motivate a person of ordinary skill in the art to modify h53AON1 to make the claimed antisense oligonucleotides with a reasonable expectation of success*

Even if the Office had established that a person of ordinary skill in the art would have selected h53AON1 as the lead compound, the second prong of the test also has not been met. The second prong of the lead compound analysis requires a determination of whether "the prior art would have supplied one of ordinary skill in the art with a reason or motivation to modify a lead compound with a reasonable expectation of success." (*Otsuka Pharmaceutical Co. Ltd., v. Sandoz, Inc.*, 678 F.3d at 1292 (2012).)

The Office relies on van Ommen et al. as teaching a genus of oligonucleotides 16-50 bases in length that are complementary to, and cause skipping of, exon 53, and selects SEQ ID NO: 29 (h53AON1), which it contends is a 18-mer oligonucleotide having a sequence identical to three nucleotides of SEQ ID NO: 195. The Office contends, "[i]t would have been obvious for one of ordinary skill in the art to make an antisense oligonucleotide of 20-31 bases" using "the sequence of h53AON1 to arrive at an oligonucleotide of 20 nucleotides and having 12 nucleotides of SEQ ID No. 195. . ." by "preparing obvious variants of h53AON1 to try to optimize the activity of the oligonucleotide. . ." using "common and efficient strategies" such as

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synthesizing and testing “longer oligonucleotides containing within them” h53AON1. (See Office Action at pages 4-5 (emphasis added).)

Applicants submit that a person of ordinary skill in the art would not have been motivated to modify h53AON1 of van Ommen et al. to arrive at the claimed morpholino antisense oligonucleotides, and certainly not with a reasonable expectation of success. Notably, none of the cited documents would have motivated one of ordinary skill in the art to *increase the length* of the 18-mer h53AON1 to 27 bases 100% complementary to the exon 53 target region +23 to +69 and, let alone select at least 12 consecutive bases of SEQ ID NO: 195 and *thymine bases* in place of uracil bases, and select a *morpholino* chemistry backbone rather than a 2'-O-methyl phosphorothioate (“2'-O-Me-PS”).²

Importantly, Applicants respectfully point out that the Office’s proposed strategy for modification of h53AON1 by lengthening it by only two bases would not result in an antisense oligonucleotide within the scope of the instant claims. To illustrate this point, Applicants provide the following alignment of h53AON1 (line 2) to SEQ ID NO: 195 (line 1).

1.	<u>CUGAAGGUGUUCUUGUACUUCAUCC</u>	SEQ ID NO: 195
2.	CUGUUGCCUCCGGUUCUG	h53AON1
3.	CUGUUGCCUCCGGUUCUGAA	h53AON1+2 bases – 20mer
4.	CUGUUGCCUCCGGUUC <u>CUGAAGGUGUUC</u>	h53AON1+9 bases = 27mer

As can be seen from above and acknowledged by the Office, h53AON1 comprises only three consecutive bases of SEQ ID NO: 195 indicated in the underlined portion of lines 1 and 2. Addition of two additional consecutive bases to h53AON1 as proposed by the Office results in a 20mer that is within the claimed length range, but such a 20mer would only comprise five consecutive bases of SEQ ID NO: 195 as illustrated in line 3 – not at least 12 consecutive bases of SEQ ID NO: 195 as required by the claims. Applicants note that to achieve an antisense oligonucleotide of the instant claims comprising, *inter alia*, at least 12 bases of SEQ ID NO: 195, the skilled artisan would need to, *inter alia*, lengthen h53AON1 by 9 bases as illustrated in the underlined portion of line 4 above. Meaning, simply lengthening h53AON1 by two bases as suggested by the Office would clearly not result in the claim requirement of at least 12 bases of

² Nor can it be found that the claimed invention would have been “obvious to try” as there are *not* a “finite number of identified, predictable solutions” such that one ordinarily skilled in the art could have pursued known potential solutions with a reasonable expectation of success. (*Examination Guidelines Update: Developments in the Obviousness Inquiry after KSR v. Teleflex*, issued by the United States Patent and Trademark Office (Federal Register, Vol. 75, No. 169: 53643, September 1, 2010); emphasis added.)

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SEQ ID NO: 195. Applicants base the remainder of the response based on modifying h53AON1 by, *inter alia*, adding 9 consecutive bases of SEQ ID NO: 195.

With regard to van Ommen et al., it cannot be said that there were a "finite number" of known, predictable solutions to the problem of designing a more efficient exon skipping antisense oligonucleotide with a reasonable expectation of success. In fact, van Ommen et al. suggest a wide variety of modifications to the antisense oligonucleotide structure with little specificity as to any individual oligonucleotide in the following:

[t]he complementary oligonucleotide generated through a method of the invention is preferably complementary to a consecutive part of between **16 and 50 nucleotides** of the exon RNA. **Different types of nucleic acid may be used** to generate the oligonucleotide. Preferably, the oligonucleotide comprises RNA, as RNA/RNA hybrids are very stable. Since one of the aims of the exon skipping technique is to direct splicing in subjects, it is preferred that the oligonucleotide RNA comprises a **modification providing the RNA with an additional property**, for instance, resistance to endonucleases and RNaseH, additional hybridization strength, increased stability (for instance, in a bodily fluid), increased or decreased flexibility, reduced toxicity, increased intracellular transport, and/or tissue-specificity, etc. Preferably, the modification comprises a 2'-O-methyl-phosphorothioate oligoribonucleotide modification.

With the advent of **nucleic acid-mimicking technology**, it has become possible to generate molecules that have a similar, preferably the same, hybridization characteristics, in kind, not necessarily in amount, as nucleic acid itself. Such equivalents are, of course, also part of the invention. **Examples of such mimics** equivalents are **peptide nucleic acid, locked nucleic acid and/or a morpholino phosphorodiamidate**. . . . **Hybrids between one or more of the equivalents among each other and/or together** with nucleic acid are, of course, also part of the invention. In a preferred embodiment, an equivalent comprises locked nucleic acid, as locked nucleic acid displays a higher target affinity and reduced toxicity and, therefore, shows a higher efficiency of exon skipping. (van Ommen et al. page 9, line 28 to page 11, line 2; emphasis added.)

van Ommen et al. also teach that "[i]t is thus not absolutely required that all the bases in the region of complementarity are capable of pairing with bases in the opposing strand... **[m]ismatches may to some extent be allowed.**" (van Ommen et al. at page 3, ll. 3-8; emphasis added.) van Ommen et al. does not require that additional bases added to the antisense oligonucleotide be complementary to exon 53. *Id.*

Thus, there are a tremendous number of possible solutions to modify h53AON1 based on the length and position of "16-50 bases," mismatches, and many possible variations at any of three "substituents" (*i.e.*, nucleobase, ribose ring and phosphate linkage). Even if one focuses on

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the nucleobase sequence, assumes the chemical backbone and internucleotide linkages are unmodified, and limits the number of possible bases to those found in RNA, as shown in h53AON1, adding a single nucleobase to a 18-mer yields 8 possible sequence combinations (A, C, G, or U added before or after the 18-mer.)³ Adding two nucleobases yields 64 possible combinations. Adding three nucleobases yields 256 combinations. Adding 9 nucleobases to obtain a 27-mer yields 2,621,440 possible combinations. And, adding 32 nucleobases to obtain a 50-mer yields 608,742,554,432,415,200,000 possible combinations.

Of course, this significantly *underestimates* the number of possible nucleobase combinations because van Ommen et al. specify "different types of nucleic acid," and is not limited to the "natural" bases A, C, G, and U found in RNA, but includes other naturally-occurring and non-naturally occurring nucleobases such as inosine, hypoxanthine, xanthine, and many others. Different types of nucleic acid also include nucleotide analogs and chemical modifications to the backbone, as all of the working examples by van Ommen et al. use 2'-O-Me-PS oligoribonucleotide modifications. Different types of nucleic acid also include "mimetics" such as peptide nucleic acids, locked nucleic acid, and morpholino phosphorodiamidates. (van Ommen et al. at page 10, ll. 11-16.) Given the incredibly large number of modifications to h53AON1 that are taught by the cited documents the only way to start from h53AON1 and modify it to arrive at the claimed antisense oligonucleotide is by the application of hindsight.

There is also no reason or motivation to specifically *increase* the length of h53AON1 as there is no teaching in van Ommen et al. with respect to the effects on exon skipping of *lengthening* (or shortening) an antisense oligonucleotide. In fact, as shown in Table 2, all of the antisense oligonucleotides with exon skipping activity are *15-24 bases in length*, and all but 3 of those are between *17 and 20 bases*, almost two thirds are either *19 or 20 bases*, and *none are 25 bases in length*. (van Ommen et al. Table 2 at page 48.) As the vast majority of the antisense oligonucleotides tested by van Ommen et al. in Table 2 are *20 bases or less* (25/30), one of ordinary skill in the art would have no reason or motivation to lengthen h53AON1 at all. In fact, one skilled in the art would be equally motivated to shorten h53AON1, as almost two thirds of

³ Assuming only the four RNA nucleobases, the number of nucleobase combinations for a particular length AON can be calculated by this formula, where "n" equals the number of bases being added to the chain: $(4^n) \times (n+1)$. This is because each additional nucleotide can be added to either end of SEQ ID NO: 29.

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the antisense oligonucleotides are either 19 or 20 bases, and the shortest antisense oligonucleotide with activity in Table 2 is 15 bases (h46AON4b).

Moreover, the Office failed to provide a reason why the skilled artisan would lengthen h53AON1. Instead, the Office merely concludes the skilled artisan would “prepare obvious variants of h53AON1 to try to optimize the activity of the oligonucleotide” and that the skilled artisan would “try” to enhance activity by “a common and efficient strategy” of synthesizing and testing “longer oligonucleotides containing within them the sequence known to have the desired activity.” Office Action at pages 4-5. The Office overlooks the fact that in Table 2 the only other antisense oligonucleotide made and tested by van Ommen et al. is h53AON2, and this antisense oligonucleotide – like h53AON1 – is an 18mer. Applicants respectfully point out that “[a] particular parameter must first be *recognized* as a *result-effective variable*, i.e., a variable which achieves a *recognized* result, before the determination of the optimum or workable ranges of said variable might be characterized as routine experimentation.” M.P.E.P. 2144.05(II)(B) (emphasis added); *see also In re Antonie*, 559 F.2d 618, 195 U.S.P.Q. 6 (CCPA 1977).

In the present case, the Office failed to satisfy its burden of providing evidence that oligonucleotide length was recognized in the prior art as a result effective variable for exon 53 skipping and activity in treatment for DMD. *See id.* Absent such evidence of recognition as a “result-effective variable[,]” it is not, therefore, routine optimization “within the skill of the artisan” to vary the length of an oligonucleotide to optimize exon 53 skipping and activity in the treatment of DMD. *See* M.P.E.P. 2144.05(II)(B); *In re Antonie*, 559 F.2d 618, 620, 195 U.S.P.Q. 6, 8-9 (C.C.P.A. 1977) (optimization of a parameter not recognized as a result-effective variable is an exception to the rule that “discovery of an optimum value of a variable in a known process is normally obvious”). Thus, the Office’s proffered rationale of routine optimization by lengthening h53AON1 does not apply.

Given the length of 16-50 bases and the many possible variations in nucleobase and backbone chemistry taught by van Ommen et al., there is *not* a “finite number” of known, predictable solutions to modifying h53AON1 such that one of ordinary skill in the art would arrive at the claimed morpholino antisense oligonucleotides of 20 to 31 bases having a base sequence 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), and having at least 12 consecutive bases of SEQ ID NO: 195 in which uracil bases are thymine bases, with a reasonable expectation of success. In fact, there is

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absolutely nothing in van Ommen et al. about selecting a morpholino chemistry backbone and thymine bases, rather than uracil bases.

iii. **High level of unpredictability in the field with no reasonable expectation of success**

Even assuming, *arguendo*, that one of ordinary skill would have selected h53AON1 of van Ommen et al. as a lead compound and would have been motivated to modify it in the particular way necessary to arrive at the subject matter of the claims, there would be no reasonable expectation of success because at the time the instant invention was made, there was a significant level of unpredictability associated with selecting specific antisense oligonucleotide sequences to induce effective dystrophin exon skipping. For example, the specification as originally filed notes that the size or length of an antisense oligonucleotide is not predictive of its efficacy (specification at page 21, lines 11-12). In addition, Applicants have found that there is no standard motif that can be blocked or masked by antisense molecules to redirect splicing (specification at page 21, lines 18-20). Applicants submit that the cited art does not provide sufficient guidance to arrive at the claimed subject matter considering the high level of unpredictability in the art.

Applicants refer the Office to van Deutekom *et al.* (2003) Nature Reviews, 4:774-783 ("van Deutekom Review"; submitted in an Information Disclosure Statement on September 22, 2017). This article is a review that generally discloses exon skipping in the dystrophin gene. The van Deutekom Review notes that interfering with exon selection for inclusion before splicing is "a process that is *not yet well understood*" (page 780, col. 1, lines 1-3, emphasis added).

Applicants also refer the Office to U.S. Patent Application Publication No. 2006/0147952 to van Ommen et al. (the '952 Publication) describe an approach in which "AONs were *empirically analyzed* for the induction of exon skipping." ('952 Publication at [0051]; emphasis added.) Such an approach relies on experience or observation and provides no indication as to what parameters are critical for the design of exon skipping antisense. As each antisense oligonucleotide must be empirically analyzed, the results are *unpredictable* as reported in Table 2 of the '952 Publication:

[t]heir different lengths and G/C contents (%) *did not correlate to their effectivity in exon skipping* (1, induced skipping, 2, no skipping). The AONs were directed to purine

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(A/G)-rich sequences as indicated by their (antisense) U/C content (%). Skipping of the target exons resulted in either an in-frame (IF) or out-of-frame (OF) transcript. (van Ommen et al. [0153], Table 2, footnote *a*; emphasis added.)

Additional evidence of unpredictability is found by analyzing the antisense sequences in Table 2 of the '952 Publication. For example, the two antisense oligonucleotides designed to induce skipping of exon 2 have overlapping nucleotide sequences:

h2AON1	cccauuuugugaauguuuucuuuu
h2AON2	uugugcauuuacccaauuugug

Despite the overlap in sequence, h2AON1 purportedly induced skipping, while h2AON2 did *not*. ('952 Publication at Table 2.) And yet for another pair of overlapping AONs, both members of the pair did purportedly induce skipping:

h29AON1	uauccucugaaugucgcauc
h29AON2	gguaauccucugaaugucgc

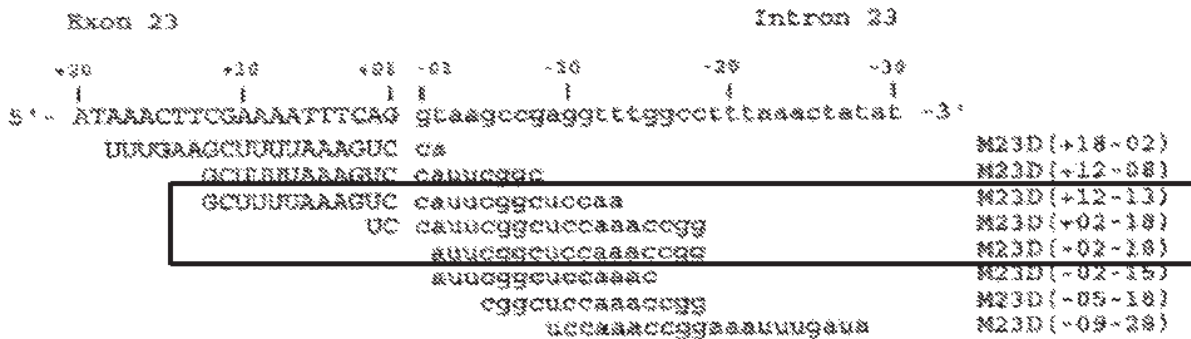
There is no explanation in the '952 Publication for these disparate results.

Much of the data in Table 2 of the '952 Publication was published in 2002 by Aartsma-Rus et al. (Neuromuscular Disorders, 12:S71-S77 (2002) ("Aartsma-Rus (2002)"; submitted in an Information Disclosure Statement on September 22, 2017). Aartsma-Rus (2002) discloses two specific oligonucleotides directed at dystrophin exon 53 and notes that there is *no correlation* between the length or sequence of the oligonucleotide and its effectiveness at inducing exon skipping. (Aartsma-Rus (2002) at page S76, col. 1, lines 43-45.) Still further, Aartsma-Rus (2002) teaches that *significant experimentation is required* to arrive at specific oligonucleotides, noting that "[w]e therefore have *no insight* into the actual position of the targeted sequence within the completely folded RNA structure. Its accessibility, and thus the effectivity of any designed AON, will therefore have to be tested *empirically* in the cells, as was done in this study." (Aartsma-Rus (2002) at page S76, col. 1, lines 4-6; emphasis added.)

Another study, co-authored by one of the Applicants, examined skipping of exon 23 from the mouse DMD gene by RT-PCR following transfection with a series of overlapping 2'-Me-O-PS AONs, as shown in the following figure. Of the antisense oligonucleotides tested, only M23D(+12-13), M23D(+02-18), and M23D(-02-18) were effective in inducing detectable exon

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skipping. (Mann et al., *J. Gene Med.*, 4(6): 644-654 (2002); submitted in an Information Disclosure Statement on September 22, 2017.)



(Mann et al. at 646.) Notably, the *shorter* antisense oligonucleotide M23D(-02-18), which is only *17 nucleotides* in length, was particularly efficient at inducing skipping and was reported to induce exon skipping at concentrations as low as 5 nM. The authors concluded that they could improve “the efficiency of the technique” by “*reduc[ing] the size* and the effective dose of the AO[N]s” examined. (Mann et al. at 644; emphasis added.)

Similar examples of unpredictability were reported by van Ommen et al. and other investigators at or near the date of Applicants' invention. In a 2005 publication the same design rationale described by van Ommen and coworkers was applied again. (Aartsma-Rus et al. Oligonucleotides, 15(4): 284-297 (2005) ("Aartsma-Rus (2005)"; submitted in an Information Disclosure Statement on September 22, 2017.) Table 1 of Aartsma-Rus (2005) provides the sequences of the antisense oligonucleotides and whether or not they induced skipping. (Aartsma-Rus (2005) at 285, first and second columns.) The following pairs of antisense oligonucleotides are found in the Table (+ and – refer to skipping ability):

h29AON10	guaguucccuccaacg	-
h29AON11	cauguaguucccucc	+
h43AON2	uuquuaaacuuuuucccauu ⁴	+

⁴ There is a discrepancy between the disclosure of Aartsma-Rus (2005) and the sequence as shown by van Ommen et al. In the 2005 publication, the sequence is shown as uuguuaacuuuuuuccauu, while in Table 2

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h43AON3	uguuaacuuuuucccauugg	-
h46AON8	gcuuuucuuuuaguugcugc	++
h46AON9	uuaguugcugcucuu	-
h48AON3	ggucuuuuauuugagcuuc	-
h48AON7	uuuauuugagcuucaaauuu	+

It is evident from these results that applying the design rationale described by van Ommen et al. is a hit-or-miss proposition in terms of whether any given antisense oligonucleotide will be capable of inducing skipping, *even in situations where the antisense oligonucleotides are very similar to each other in terms of nucleotide sequence, and other variables concerning the chemical backbone are fixed*. All of the antisense oligonucleotides described in the study “contain 2’-O-methyl RNA and full-length phosphorothioate (PS) backbones.” (Aartsma-Rus (2005) at 285.) None of the antisense oligonucleotides disclosed were longer than 24 nucleotides, and the majority of the antisense oligonucleotides were 20 nucleotides in length or shorter. (Aartsma-Rus at Table 1.) None of these antisense oligonucleotides include non-natural bases. Given the common chemical modifications of these antisense oligonucleotides, the data reported in this paper demonstrates the unpredictable impact that length and nucleotide composition make with respect to efficiency in inducing exon skipping.

The recognition of the lack of predictability in the field of exon skipping continued beyond 2005. A 2007 paper co-authored by van Ommen co-inventors Aartsma-Rus and van Deutekom states that “several years after the first attempts at dystrophin exon skipping with AOs [antisense oligonucleotides], *there are still no clear rules to guide investigators in their design*, and in mouse and human muscle cells *in vitro there is great variability for different targets and exons*.” (Arechavala-Gomez et al. Hum. Gene Ther., 18(9): 798-810, 807 (2007); submitted in an Information Disclosure Statement on September 22, 2017; emphasis added.)

And again in 2009 van Ommen and co-workers wrote that while existing software programs can facilitate design, “in general *a trial and error procedure* is still involved to

of van Ommen et al. it shown as above having a sequence of "ccc" toward the 3' end of the AON. It is assumed the latter is correct as it corresponds to the sequence of h43AON3.

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identify potent AONs.” (Aartsma-Rus et al., *Mol. Ther.*, 17(3):548-553 (2009) at 548; submitted in an Information Disclosure Statement on September 22, 2017; emphasis added.)

Evidence that selecting specific antisense oligonucleotide sequences to induce effective dystrophin exon skipping remains an unpredictable exercise is also found in a 2011 publication by Wu *et al.* (2011) *PLoS One*, 6(5): e19906 (submitted in an Information Disclosure Statement on September 22, 2017). Although Wu *et al.* is evidence developed after the instant filing date, the level of unpredictability in the art directly relates to whether the results obtained with any specific species would be unexpected and courts have held that it is not “improper to conduct additional experiments and provide later-obtained data in support of patent validity.” *Knoll Pharm. Co., Inc. v. Teva Pharms. USA, Inc.*, 367 F.3d 1381, 1385 (Fed. Cir. 2004). Evidence of the lack of predictability of in the field is relevant to the non-obviousness of the claimed antisense oligonucleotides over the cited art.

Wu *et al.* describe a systematic approach for identifying antisense oligonucleotides of high efficacy in inducing dystrophin exon skipping. Wu *et al.* designed 25 antisense oligonucleotides (AOs) to cover more than two thirds of exon 50 of the human dystrophin gene and the two flanking intron sequences. Wu *et al.* determined the efficiency of AO-induced skipping of exon 50 by comparing the activity of a series of AOs. Table 1 on page 4 of the publication summarizes all the AOs tested, including both 2'-O-methyl phosphorothioate and morpholino antisense oligonucleotides, as well as their reported activity in two assays. The exon skipping effect was determined using both a GFP reporter cell line with GFP expression coupled to exon 50 skipping and normal human myoblasts.

As shown in Table 1, Wu *et al.* tested AOs having a common 5' or 3' termini, but varied in length. Shown below is an excerpt from Table 1 of Wu *et al.*

NE50 AO2PS	-19--1	5'-CUUUACAGAAAAGCAUAC-3'	19 bp	-	-	N/D
NE50 AO3PS	-19+1	6'-UCUUUACAGAAAAGCAUAC-3'	20 bp	-	-	N/D
NE50 AO4PS	-19+3	5'-CCUCUUUACAGAAAAGCAUAC-3'	22 bp	4%	3%	N/D
NE50 AO5PS	-19+8	5'-AACUUCUCUUUACAGAAAAGCAUAC-3'	27 bp	21%	29%	N/D
NE50 AO6PS	-19+13	5'-CUUCUAAUCUCCUUUUACAGAAAAGCAUAC-3'	32 bp	3%	<1%	N/D

Each of these AOs target exon 50 starting at position (-19) and ending at position (-1), (+1), (+3), (+8) and (+13), respectively, and the oligonucleotides overlap at the 3' end. These AOs varied in length from 19 to 32 bases and the data shows that increasing AO length does not

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necessarily increase exon skipping activity and there is no reasonable expectation of success in increasing AO length to obtain increased exon skipping activity. For example, the 19- and 20-mer AOs hE50 AO2PS and hE50AO3PS were inactive. Increasing the length to 22 and 27 bases (hE50 AO4PS and hE50 AO5PS, respectively) resulted in increased activity, but a further increase to 32 bases (hE50 AO6PS) decreased activity significantly. Specifically, hE50 AO5PS is 5 nucleotides longer than hE50 AO4PS, but the level of GFP of hE50 AO5PS is 17% higher with respect to GFP assay and 26% higher with respect to human myoblasts. hE50 AO5PS is 5 nucleotides shorter than hE50 AO6PS, but the level of GFP of hE50 AO5PS is 18% higher with respect to GFP and 28% higher with respect to human myoblasts.

The data provided in Table 1 also demonstrate that when hE50 AO4PS (-19+3) was extended five nucleotides in length to hE50A AO5PS (-19+8), activity was increased. Notably, however, the addition of yet another five nucleotides to hE50 AO6PS (-19+13) essentially eliminated the activity.

In yet another example, a relatively short oligonucleotide (hE50 AO19PS; +97-5) at the 3' end of the exon showed low activity (3%) with respect to GFP, and activity did not increase when the oligonucleotide was lengthened by five or nine nucleotides at the 5' end (hE50 AO20PS and hE50 AO21PS, respectively) or by five nucleotides in the 3' direction (hE50 AO16PS). These four antisense oligonucleotides showed no activity in the human myoblasts. Thus, Wu *et al.* demonstrate that increasing or decreasing AO length results in unpredictable effects on exon skipping.

Importantly, the Patent Trial and Appeal Board (PTAB) in Interference No. 106,007 (“the ‘007 interference”) concerning exon 53 antisense oligonucleotides for DMD held that the field of antisense oligonucleotides for exon skipping for DMD was unpredictable at the time the instant application was filed. Its decision was based on the foregoing evidence and expert testimony. *See* Decision on Motions in Interference No. 106,007 (exon 53) dated May 12, 2016 (decision final upon withdrawal of CAFC Appeal No. 2016-2262; Decision on Motions previously submitted in an Information Disclosure Statement on September 22, 2017). Specifically, the PTAB determined that sequence length of antisense oligonucleotides that would maintain exon skipping was substantially unpredictable at the time US Application No. 11/233,495 was filed by Academisch Ziekenhuis Leiden (“AZL”). *See id.* at page 5, line 26 to page 6, line 3. Applicants note that the ‘495 application claims priority to the van Ommen *et al.* PCT application presently cited by the Office. In its Decision, the PTAB

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considered the foregoing evidence as representative of the state of the art with Exhibits 2010 and 2015 in Interference 106,007 corresponding to Aartsma-Rus and Wu *et al.*, submitted herewith as Appendices A and C, respectively. Unpredictability in this art was determined by the PTAB to have existed at the time of the instant invention (and years afterwards).

Upon consideration of this evidence, the PTAB stated “[t]he evidence indicates that at the time AZL filed its application, the identification of AONs that will cause exon skipping was generally thought to be unpredictable. One of the significant factors causing that unpredictability is the effect of the number of nucleobases present in the AON.” (Decision on Motions at page 17 (emphasis added)). In particular, the relationship between length of a base sequence and the ability of an antisense oligonucleotide to induce exon skipping was considered by the PTAB.

Despite the unpredictability in the art, the PTAB found obvious a 20mer AON based on SEQ ID NO: 193 over a completely overlapping 18mer (h53AON1). In this particular circumstance, the PTAB found that “a degree of exon skipping capability would likely be maintained due to a change in a *small number of complementary nucleobases* of an AON known to cause skipping” and, therefore, concluded “[i]t would have been obvious, for example, to add the *two* complementary nucleobases dictated by the known sequence of exon 53 to either end of h53AON1 with a reasonable expectation that the resultant 20 base AON would cause exon skipping.” *Id.* at pages 41-42 (emphasis added).

In contrast to the narrow issue considered by the PTAB described above, the PTAB does not support a determination of obviousness of the instant claims. The PTAB’s determination of unpredictability still applies. And to arrive at the instantly claimed antisense oligonucleotides, a person of ordinary skill would have to modify h53AON1 by adding at least *9 bases* (and would have to do so with a reasonably expectation of success). Such a modification in length cannot be said to be predictable under the Decision in the ‘007 interference. Accordingly, it would not have been obvious to extend h53AON1 by 9 bases at least because of the highly degree of unpredictability discussed above, and the Office failed to provide evidence to the contrary.

Furthermore, similar to the Office’s assertion, AZL argued that upon identification of h53AON1, “one skilled in the art would have investigated extended complementary sequences with the expectation that the longer sequences would bind and cause skipping.” *Id.* The PTAB did not find this argument persuasive at least because AZL failed to provide any

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evidence to support the basis for this expectation. *Id.* at page 18. Like AZL, the Office failed to provide evidence to support this argument. *See* Office Action at page 5. Accordingly, Applicants urge the Office to adopt the PTAB's determination of unpredictability in the field of exon skipping for DMD.

In summary, the van Deutekom Review, Aartsma-Rus and Wu *et al.* references, along with the Decision on Motions in the '007 interference, serve to illustrate the unpredictability associated with selecting *specific* antisense oligonucleotides that are effective for inducing skipping of dystrophin exons. Accordingly, the Office failed to establish a *prima facie* case of obviousness with respect to the predictability of the outcome in combining teachings of van Ommen *et al.* and Koenig *et al.* in the manner proposed to arrive at the claimed invention.

In view of the preceding remarks, Applicants submit that the Office failed to establish a *prima facie* case of obviousness based on the cited art. As such, Applicants respectfully request reconsideration and withdrawal of this obviousness rejection.

Double Patenting

Claims 2 and 3 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-36 of U.S. Patent No. 8,455,636. Applicants respectfully traverse this rejection.

The Office asserts "the instant claims and the claims of the patent are drawn to antisense oligonucleotides having at least 17 consecutive bases of SEQ ID No. 193." Office Action at page 6. However, Applicants note the instant claims are drawn to antisense oligonucleotide having 20-31 bases and comprising at least 12 consecutive bases of SEQ ID NO: 195. Moreover, the '636 patent is directed to an antisense oligonucleotide comprising 20-50 bases and at least 20 consecutive bases of SEQ ID NO: 193. As such, Applicants point out that there is only a 2 base overlap between SEQ ID NOs: 193 of the '636 Patent and SEQ ID NO: 195 of the instant claims. Accordingly, Applicants respectfully request that the Office consider withdrawing the instant rejection in view of these facts and the foregoing remarks.

Claims 2 and 3 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-25 of U.S. Patent No. 8,232,384. Applicants respectfully request clarification of this rejection. Specifically, The Office asserts

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“the instant claims and the claims of the patent are drawn to antisense oligonucleotides having at least 17 consecutive bases of SEQ ID No. 193.” Office Action at page 7. However, Applicants note the instant claims are drawn to antisense oligonucleotide having 21-30 bases and comprising at least 12 consecutive bases of SEQ ID NO: 195. Moreover, the ‘384 patent is directed to an antisense oligonucleotide *consisting of* SEQ ID NO: 195. Accordingly, Applicants respectfully request clarification.

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CONCLUSION

In view of the foregoing, Applicants respectfully submit that the pending claims are in condition for allowance. If a telephone conversation with Applicants' attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 217-4626. If a fee is due with this submission, please charge our Deposit Account No. 12-0080 under Order No. AVN-008CN41, from which the undersigned is authorized to draw

Dated: January 5, 2018

Respectfully submitted,
Electronic signature: /Amy E. Mandragouras,
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/705,172	09/14/2017	Stephen Donald WILTON	4140,01500A9	2879

153767 7590 04/04/2018
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 UNITED STATES OF AMERICA

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1674	

MAIL DATE	DELIVERY MODE
04/04/2018	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary

Application No.

15/705,172

Applicant(s)

WILTON et al.

Examiner

KIMBERLY CHONG

Art Unit

1674

AIA Status

No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 01/05/2018.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.
- 2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

- 5) ☒ Claim(s) 2-3 is/are pending in the application.
 5a) Of the above claim(s) ____ is/are withdrawn from consideration.
- 6) ☐ Claim(s) ____ is/are allowed.
- 7) ☒ Claim(s) 2-3 is/are rejected.
- 8) ☐ Claim(s) ____ is/are objected to.
- 9) ☐ Claim(s) ____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

- 10) ☐ The specification is objected to by the Examiner.
- 11) ☐ The drawing(s) filed on ____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

- a) ☐ All b) ☐ Some** c) ☐ None of the:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. ____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☐ Notice of References Cited (PTO-892)
- 2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)
 Paper No(s)/Mail Date 01/05/2018
- 3) ☒ Interview Summary (PTO-413)
 Paper No(s)/Mail Date 03/26/2018
- 4) ☐ Other: ____.

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Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Status of Application/Amendment/Claims

Claims 2 and 3 are pending and currently under examination.

Information Disclosure Statement

The submission of the Information Disclosure Statements on 01/05/2018 is in compliance with 37 CFR 1.97. The information disclosure statement has been considered by the examiner and signed copies have been placed in the file.

Response to Arguments

Claim Rejections - 35 USC § 103

The rejection of claims 2 and 3 under pre-AIA 35 U.S.C. 103(a) as being obvious over van Ommen (WO2004/083432 cited on IDS filed 09/22/2017) and Koenig et al. (Nature 338, 509 - 511 06 April 1989 cited on IDS filed 09/22/2017) is withdrawn in response to Applicant's argument that one of skill in the art would not have been motivated to make the claimed oligonucleotide from h53AON1 taught by van Ommen.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11

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F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to

<http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp>.

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The rejection of claims 2 and 3 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-36 of U.S. Patent No. 8,455,636 is withdrawn in response to Applicant's arguments.

The rejection of claims 2 and 3 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-25 of U.S. Patent No. 8,232,384 is maintained for the reasons of record.

Patent '384 are drawn to an antisense oligonucleotide targeted to annealing site H53A (+23+47) and consisting of SEQ ID No. 195 which is 25 nucleotides in length. The instant claims are drawn to an antisense oligonucleotide targeted to annealing site H53A (+23+47) having 20-31 bases comprising at least 12 consecutive bases of SEQ ID No. 195 but could also encompass 25 nucleotides of SEQ ID No. 195. Therefore the instant claims and the claims of the patent are not patentably distinct from each other.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of

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the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action. Any inquiry concerning this communication or earlier communications from the examiner should be directed to KIMBERLY CHONG **whose telephone number is** (571)272-3111. The examiner can normally be reached Monday thru Friday 9-5 pm.

If attempts to reach the examiner by telephone are unsuccessful please contact the SPE for 1674 Ram Shukla at 571-272-07350735. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see <http://pair-direct.uspto.gov>.

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For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Primary Examiner
Art Unit 1674

EXHIBIT 23

5234739

**THE UNITED STATES OF AMERICA****TO ALL TO WHOM THESE PRESENTS SHALL COME:****UNITED STATES DEPARTMENT OF COMMERCE****United States Patent and Trademark Office**

April 25, 2022

**THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE
RECORDS OF THIS OFFICE OF THE FILE WRAPPER AND CONTENTS
OF:**

APPLICATION NUMBER: 16/112,453**FILING DATE:** August 24, 2018**PATENT NUMBER:** 10,266,827**ISSUE DATE:** April 23, 2019

**By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office**



Wanda Montgomery
Wanda Montgomery
Certifying Officer

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	4140.01500B1
		Application Number	
Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		
The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76. This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.			

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Stephen	Donald	WILTON		
Residence Information (Select One) <input type="radio"/> US Residency <input checked="" type="radio"/> Non US Residency <input type="radio"/> Active US Military Service					
City	Applecross		Country of Residence ⁱ	AU	
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Address 1		18 Spey Road			
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City	Applecross		State/Province		
Postal Code	6153		Country ⁱ	AU	
Inventor 2					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Sue		FLETCHER		
Residence Information (Select One) <input type="radio"/> US Residency <input checked="" type="radio"/> Non US Residency <input type="radio"/> Active US Military Service					
City	Bayswater		Country of Residence ⁱ	AU	
Mailing Address of Inventor:					
Address 1		14 Roberts Street			
Address 2					
City	Bayswater		State/Province		
Postal Code	6053		Country ⁱ	AU	
Inventor 3					Remove
Legal Name					

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	4140.01500B1
		Application Number	
Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		

Prefix	Given Name	Middle Name	Family Name	Suffix
	Graham		MCCLOREY	
Residence Information (Select One) <input type="radio"/> US Residency <input checked="" type="radio"/> Non US Residency <input type="radio"/> Active US Military Service				
City	Bayswater	Country of Residence ⁱ	AU	
Mailing Address of Inventor:				
Address 1		8 Digwood Close		
Address 2				
City	Bayswater	State/Province		
Postal Code	6053	Country ⁱ	AU	
All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the Add button.				
<input type="button" value="Add"/>				

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).	
<input type="checkbox"/> An Address is being provided for the correspondence information of this application.	
Customer Number	153767
Email Address	<input type="button" value="Add Email"/> <input type="button" value="Remove Email"/>

Application Information:

Title of the Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		
Attorney Docket Number	4140.01500B1	Small Entity Status Claimed	<input checked="" type="checkbox"/>
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	22	Suggested Figure for Publication (if any)	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	4140.01500B1
		Application Number	
Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		

Publication Information:

<input type="checkbox"/>	Request Early Publication (Fee required at time of Request 37 CFR 1.219)
<input type="checkbox"/>	Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.			
Please Select One:	<input checked="" type="radio"/> Customer Number	<input type="radio"/> US Patent Practitioner	<input type="radio"/> Limited Recognition (37 CFR 11.9)
Customer Number	153767		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending	Remove			
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
	Continuation of	15274772	2016-09-23		
Prior Application Status	Patented	Remove			
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
15274772	Continuation of	14740097	2015-06-15	9605262	2017-03-28
Prior Application Status	Abandoned	Remove			
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
14740097	Continuation of	13741150	2013-01-14		
Prior Application Status	Abandoned	Remove			
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
13741150	Continuation of	13168857	2011-06-24		

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number		4140.01500B1	
		Application Number			
Title of Invention		ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF			

Prior Application Status		Patented		Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13168857	Continuation of	12837359	2010-07-15	8232384	2012-07-31

Prior Application Status		Patented		Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12837359	Continuation of	11570691	2008-01-15	7807816	2010-10-05

Prior Application Status		Expired		Remove	
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
11570691	a 371 of international	PCT/AU2005/000943	2005-06-28		

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Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)ⁱ the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

		Remove	
Application Number	Country ⁱ	Filing Date (YYYY-MM-DD)	Access Code ^j (if applicable)
2004903474	AU	2004-06-28	

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.

☐ NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	4140.01500B1
		Application Number	
Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.


☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	4140.01500B1
		Application Number	
Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.			
Applicant 1			
If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.			
			
<input checked="" type="radio"/> Assignee		<input type="radio"/> Legal Representative under 35 U.S.C. 117	
<input type="radio"/> Person to whom the inventor is obligated to assign.		<input type="radio"/> Person who shows sufficient proprietary interest	
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:			
Name of the Deceased or Legally Incapacitated Inventor:			
If the Applicant is an Organization check here. <input checked="" type="checkbox"/>			
Organization Name	The University of Western Australia		
Mailing Address Information For Applicant:			
Address 1	35 Stirling Highway		
Address 2			
City	Crawley	State/Province	
Country ¹	AU	Postal Code	6009
Phone Number		Fax Number	
Email Address			
Additional Applicant Data may be generated within this form by selecting the Add button.			

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	4140.01500B1
		Application Number	
Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		

Assignee 1			
Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.			
If the Assignee or Non-Applicant Assignee is an Organization check here. <input checked="" type="checkbox"/>			
Organization Name	The University of Western Australia		
Mailing Address Information For Assignee including Non-Applicant Assignee:			
Address 1	35 Stirling Highway		
Address 2			
City	Crawley	State/Province	
Country ⁱ	AU	Postal Code	6009
Phone Number		Fax Number	
Email Address			
Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.			

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). However, if this Application Data Sheet is submitted with the **INITIAL** filing of the application **and** either box A or B is **not** checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/John M. Covert, #38,759/		Date (YYYY-MM-DD)	2018-08-24	
First Name	John	Last Name	Covert	Registration Number	38759
Additional Signature may be generated within this form by selecting the Add button.					

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	4140.01500B1
		Application Number	
Title of Invention	ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF		

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING
AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application is a continuation of U.S. Patent Application No. 15/274,772, filed September 23, 2016, now pending, which application is a continuation of U.S. Patent Application No. 14/740,097, filed June 15, 2015, now issued as U.S. Patent No. 9,605,262, which application is a continuation of U.S. Patent Application No. 13/741,150, filed January 14, 2013, now abandoned, which application is a continuation of U.S. Patent
10 Application No. 13/168,857, filed June 24, 2011, now abandoned, which application is a continuation of U.S. Patent Application No. 12/837,359, filed July 15, 2010, now issued as U.S. Patent No. 8,232,384, which application is a continuation of U.S. Patent Application No. 11/570,691, filed January 15, 2008, now issued as U.S. Patent No. 7,807,816, which application is a 35 U.S.C. § 371 National Phase Application of PCT/AU2005/000943, filed
15 June 28, 2005, which claims priority to Australian Patent Application No. 2004903474, filed June 28, 2004; which applications are each incorporated herein by reference in their entireties.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

20 This invention was made with government support under grant number R01 NS044146 awarded by the National Institutes of Health. The government has certain rights in the invention.

STATEMENT REGARDING SEQUENCE LISTING

25 The Sequence Listing associated with the application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 4140.01500B1_SL.txt. The text file is 62,078 bytes, was created on August 23, 2018 and is being submitted electronically via EFS-Web.

FIELD OF THE INVENTION

The present invention relates to novel antisense compounds and compositions suitable for facilitating exon skipping. It also provides methods for inducing exon skipping using the novel antisense compounds as well as therapeutic compositions
5 adapted for use in the methods of the invention.

BACKGROUND ART

Significant effort is currently being expended researching methods for suppressing or compensating for disease-causing mutations in genes. Antisense technologies are being developed using a range of chemistries to affect gene expression at a
10 variety of different levels (transcription, splicing, stability, translation). Much of that research has focused on the use of antisense compounds to correct or compensate for abnormal or disease-associated genes in a myriad of different conditions.

Antisense molecules are able to inhibit gene expression with exquisite specificity and because of this many research efforts concerning oligonucleotides as
15 modulators of gene expression have focused on inhibiting the expression of targeted genes such as oncogenes or viral genes. The antisense oligonucleotides are directed either against RNA (sense strand) or against DNA where they form triplex structures inhibiting transcription by RNA polymerase II. To achieve a desired effect in specific gene down-regulation, the oligonucleotides must either promote the decay of the targeted mRNA or
20 block translation of that mRNA, thereby effectively preventing *de novo* synthesis of the undesirable target protein.

Such techniques are not useful where the object is to up-regulate production of the native protein or compensate for mutations which induce premature termination of translation such as nonsense or frame-shifting mutations. Furthermore, in cases where a
25 normally functional protein is prematurely terminated because of mutations therein, a means for restoring some functional protein production through antisense technology has been shown to be possible through intervention during the splicing processes (Sierakowska H, *et al.*, (1996) Proc Natl Acad Sci USA 93, 12840-12844; Wilton SD, *et al.*, (1999)

Neuromusc Disorders 9, 330-338; van Deutekom JC *et al.*, (2001) Human Mol Genet 10, 1547-1554). In these cases, the defective gene transcript should not be subjected to targeted degradation so the antisense oligonucleotide chemistry should not promote target mRNA decay.

5 In a variety of genetic diseases, the effects of mutations on the eventual expression of a gene can be modulated through a process of targeted exon skipping during the splicing process. The splicing process is directed by complex multi-particle machinery that brings adjacent exon-intron junctions in pre-mRNA into close proximity and performs cleavage of phosphodiester bonds at the ends of the introns with their subsequent
10 reformation between exons that are to be spliced together. This complex and highly precise process is mediated by sequence motifs in the pre-mRNA that are relatively short semi-conserved RNA segments to which bind the various nuclear splicing factors that are then involved in the splicing reactions. By changing the way the splicing machinery reads or recognises the motifs involved in pre-mRNA processing, it is possible to create
15 differentially spliced mRNA molecules. It has now been recognised that the majority of human genes are alternatively spliced during normal gene expression, although the mechanisms invoked have not been identified. Using antisense oligonucleotides, it has been shown that errors and deficiencies in a coded mRNA could be bypassed or removed from the mature gene transcripts.

20 In nature, the extent of genetic deletion or exon skipping in the splicing process is not fully understood, although many instances have been documented to occur, generally at very low levels (Sherrat TG, *et al.*, (1993) Am J Hum Genet 53, 1007-1015). However, it is recognised that if exons associated with disease-causing mutations can be specifically deleted from some genes, a shortened protein product can sometimes be
25 produced that has similar biological properties of the native protein or has sufficient biological activity to ameliorate the disease caused by mutations associated with the target exon (Lu QL, *et al.*, (2003) Nature Medicine 9, 1009-1014; Aartsma-Rus A *et al.*, (2004) Am J Hum Genet 74: 83-92).

This process of targeted exon skipping is likely to be particularly useful in long genes where there are many exons and introns, where there is redundancy in the genetic constitution of the exons or where a protein is able to function without one or more particular exons (*e.g.* with the dystrophin gene, which consists of 79 exons; or possibly
5 some collagen genes which encode for repeated blocks of sequence or the huge nebulin or titin genes which are comprised of ~80 and over 370 exons, respectively).

Efforts to redirect gene processing for the treatment of genetic diseases associated with truncations caused by mutations in various genes have focused on the use of antisense oligonucleotides that either: (1) fully or partially overlap with the elements
10 involved in the splicing process; or (2) bind to the pre-mRNA at a position sufficiently close to the element to disrupt the binding and function of the splicing factors that would normally mediate a particular splicing reaction which occurs at that element (*e.g.*, binds to the pre-mRNA at a position within 3, 6, or 9 nucleotides of the element to be blocked).

For example, modulation of mutant dystrophin pre-mRNA splicing with
15 antisense oligoribonucleotides has been reported both *in vitro* and *in vivo*. In one type of dystrophin mutation reported in Japan, a 52-base pair deletion mutation causes exon 19 to be removed with the flanking introns during the splicing process (Matsuo *et al.*, (1991) J Clin Invest., 87:2127-2131). An *in vitro* minigene splicing system has been used to show that a 31-mer 2'-O-methyl oligoribonucleotide complementary to the 5' half of the deleted
20 sequence in dystrophin Kobe exon 19 inhibited splicing of wild-type *pre-mRNA* (Takeshima *et al.* (1995), J. Clin. Invest., 95, 515-520). The same oligonucleotide was used to induce exon skipping from the native dystrophin gene transcript in human cultured lymphoblastoid cells.

Dunckley *et al.*, (1997) Nucleosides & Nucleotides, 16, 1665-1668
25 described *in vitro* constructs for analysis of splicing around exon 23 of mutated dystrophin in the *mdx* mouse mutant, a model for muscular dystrophy. Plans to analyse these constructs *in vitro* using 2' modified oligonucleotides targeted to splice sites within and adjacent to mouse dystrophin exon 23 were discussed, though no target sites or sequences were given.

2'-O-methyl oligoribonucleotides were subsequently reported to correct dystrophin deficiency in myoblasts from the *mdx* mouse from this group. An antisense oligonucleotide targeted to the 3' splice site of murine dystrophin intron 22 was reported to cause skipping of the mutant exon as well as several flanking exons and created a novel in-
5 frame dystrophin transcript with a novel internal deletion. This mutated dystrophin was expressed in 1-2% of antisense treated *mdx* myotubes. Use of other oligonucleotide modifications such as 2'-O-methoxyethyl phosphodiester are described (Dunckley *et al.* (1998) Human Mol. Genetics, 5, 1083-90).

Thus, antisense molecules may provide a tool in the treatment of genetic
10 disorders such as Duchenne Muscular Dystrophy (DMD). However, attempts to induce exon skipping using antisense molecules have had mixed success. Studies on dystrophin exon 19, where successful skipping of that exon from the dystrophin pre-mRNA was achieved using a variety of antisense molecules directed at the flanking splice sites or motifs within the exon involved in exon definition as described by Errington *et al.* (2003) J
15 Gen Med 5, 518-527".

In contrast to the apparent ease of exon 19 skipping, the first report of exon 23 skipping in the *mdx* mouse by Dunckley *et al.*, (1998) is now considered to be reporting only a naturally occurring revertant transcript or artefact rather than any true antisense activity. In addition to not consistently generating transcripts missing exon 23, Dunckley
20 *et al.*, (1998) did not show any time course of induced exon skipping, or even titration of antisense oligonucleotides, to demonstrate dose dependent effects where the levels of exon skipping corresponded with increasing or decreasing amounts of antisense oligonucleotide. Furthermore, this work could not be replicated by other researchers.

The first example of specific and reproducible exon skipping in the *mdx*
25 mouse model was reported by Wilton *et al.*, (1999) Neuromuscular Disorders 9, 330-338. By directing an antisense molecule to the donor splice site, consistent and efficient exon 23 skipping was induced in the dystrophin mRNA within 6 hours of treatment of the cultured cells. Wilton *et al.*, (1999), also describe targeting the acceptor region of the mouse dystrophin pre-mRNA with longer antisense oligonucleotides and being unable to repeat

the published results of Dunckley *et al.*, (1998). No exon skipping, either 23 alone or multiple removal of several flanking exons, could be reproducibly detected using a selection of antisense oligonucleotides directed at the acceptor splice site of intron 22.

While the first antisense oligonucleotide directed at the intron 23 donor splice site induced consistent exon skipping in primary cultured myoblasts, this compound was found to be much less efficient in immortalized cell cultures expressing higher levels of dystrophin. However, with refined targeting and antisense oligonucleotide design, the efficiency of specific exon removal was increased by almost an order of magnitude (see Mann CJ *et al.*, (2002) J Gen Med 4, 644-654).

Thus, there remains a need to provide antisense oligonucleotides capable of binding to and modifying the splicing of a target nucleotide sequence. Simply directing the antisense oligonucleotides to motifs presumed to be crucial for splicing is no guarantee of the efficacy of that compound in a therapeutic setting.

SUMMARY OF THE INVENTION

The present invention provides antisense molecule compounds and compositions suitable for binding to RNA motifs involved in the splicing of pre-mRNA that are able to induce specific and efficient exon skipping and a method for their use thereof.

The choice of target selection plays a crucial role in the efficiency of exon skipping and hence its subsequent application of a potential therapy. Simply designing antisense molecules to target regions of pre-mRNA presumed to be involved in splicing is no guarantee of inducing efficient and specific exon skipping. The most obvious or readily defined targets for splicing intervention are the donor and acceptor splice sites although there are less defined or conserved motifs including exonic splicing enhancers, silencing elements and branch points.

The acceptor and donor splice sites have consensus sequences of about 16 and 8 bases respectively (see Figure 1 for schematic representation of motifs and domains involved in exon recognition, intron removal and the splicing process).

According to a first aspect, the invention provides antisense molecules capable of binding to a selected target to induce exon skipping.

For example, to induce exon skipping in exons 3 to 8, 10 to 16, 19 to 40, 42 to 44, 46, 47, and 50 to 53 in the Dystrophin gene transcript the antisense molecules are
5 preferably selected from the group listed in Table 1A.

In a further example, it is possible to combine two or more antisense oligonucleotides of the present invention together to induce multiple exon skipping in exons 19-20, and 53. This is a similar concept to targeting of a single exon. A combination or "cocktail" of antisense oligonucleotides are directed at adjacent exons to
10 induce efficient exon skipping.

In another example, to induce exon skipping in exons 19-20, 31, 34 and 53 it is possible to improve exon skipping of a single exon by joining together two or more antisense oligonucleotide molecules. This concept is termed by the inventor as a "weasel", an example of a cunningly designed antisense oligonucleotide. A similar concept has been
15 described in Aartsma-Rus A *et al.*, (2004) Am J Hum Genet 74: 83-92).

According to a second aspect, the present invention provides antisense molecules selected and or adapted to aid in the prophylactic or therapeutic treatment of a genetic disorder comprising at least an antisense molecule in a form suitable for delivery to a patient.

20 According to a third aspect, the invention provides a method for treating a patient suffering from a genetic disease wherein there is a mutation in a gene encoding a particular protein and the affect of the mutation can be abrogated by exon skipping, comprising the steps of: (a) selecting an antisense molecule in accordance with the methods described herein; and (b) administering the molecule to a patient in need of such treatment.

25 The invention also addresses the use of purified and isolated antisense oligonucleotides of the invention, for the manufacture of a medicament for treatment of a genetic disease.

The invention further provides a method of treating a condition characterised by Duchenne muscular dystrophy, which method comprises administering to

a patient in need of treatment an effective amount of an appropriately designed antisense oligonucleotide of the invention, relevant to the particular genetic lesion in that patient.

Further, the invention provides a method for prophylactically treating a patient to prevent or at least minimise Duchene muscular dystrophy, comprising the step of: administering to

5 the patient an effective amount of an antisense oligonucleotide or a pharmaceutical composition comprising one or more of these biological molecules.

The invention also provides kits for treating a genetic disease, which kits comprise at least a antisense oligonucleotide of the present invention, packaged in a suitable container and instructions for its use.

10 Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following figures.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Schematic representation of motifs and domains involved in exon
15 recognition, intron removal and the splicing process (SEQ ID NOS: 213 and 214).

Figure 2. Diagrammatic representation of the concept of antisense oligonucleotide
induced exon skipping to by-pass disease-causing mutations (not drawn to
scale). The hatched box represents an exon carrying a mutation that
20 prevents the translation of the rest of the mRNA into a protein. The solid black bar represents an antisense oligonucleotide that prevents inclusion of that exon in the mature mRNA.

Figure 3 Gel electrophoresis showing differing efficiencies of two antisense
molecules directed at exon 8 acceptor splice site. The preferred compound
25 [H8A(-06+18)] induces strong and consistent exon skipping at a transfection concentration of 20 nanomolar in cultured normal human muscle cells. The less preferred antisense oligonucleotide [H8A(-06+14)] also induces efficient exon skipping, but at much higher concentrations. Other antisense

oligonucleotides directed at exon 8 either only induced lower levels of exon skipping or no detectable skipping at all (not shown).

Figure 4

Gel electrophoresis showing differing efficiencies of two antisense molecules directed at internal domains within exon 7, presumably exon splicing enhancers. The preferred compound [H7A(+45+67)] induces strong and consistent exon skipping at a transfection concentration of 20 nanomolar in cultured human muscle cells. The less preferred antisense oligonucleotide [H7A(+2+26)] induces only low levels of exon skipping at the higher transfection concentrations. Other antisense oligonucleotides directed at exon 7 either only induced lower levels of exon skipping or no detectable skipping at all (not shown).

Figure 5

Gel electrophoresis showing an example of low efficiency exon 6 skipping using two non-preferred antisense molecules directed at human exon 6 donor splice site. Levels of induced exon 6 skipping are either very low [H6D(+04-21)] or almost undetectable [H6D(+18-04)]. These are examples of non-preferred antisense oligonucleotides to demonstrate that antisense oligonucleotide design plays a crucial role in the efficacy of these compounds.

Figure 6

Gel electrophoresis showing strong and efficient human exon 6 skipping using an antisense molecules [H6A(+69+91)] directed at an exon 6 internal domain, presumably an exon splicing enhancer. This preferred compound induces consistent exon skipping at a transfection concentration of 20 nanomolar in cultured human muscle cells.

Figure 7

Gel electrophoresis showing strong human exon 4 skipping using an antisense molecule H4A(+13+32) directed at an exon 6 internal domain, presumably an exon splicing enhancer. This preferred compound induces strong and consistent exon skipping at a transfection concentration of 20 nanomolar in cultured human muscle cells,

- Figure 8A Gel electrophoresis showing strong human exon 12 skipping using antisense molecule H12A(+52+75) directed at exon 12 internal domain.
- Figure 8B Gel electrophoresis showing strong human exon 11 skipping using antisense molecule H11A(+75+97) directed at an exon 11 internal domain.
- 5 Figure 9A Gel electrophoresis showing strong human exon 15 skipping using antisense molecules H15A(+48+71) and H15A(-12+19) directed at an exon 15 internal domain.
- Figure 9B Gel electrophoresis showing strong human exon 16 skipping using antisense molecules H16A(-12+19) and H16A(-06+25).
- 10 Figure 10 Gel electrophoresis showing human exon 19/20 skipping using antisense molecules H20A(+44+71) and H20A(+149+170) directed at an exon 20 and a "cocktail" of antisense oligonucleotides H19A(+35+65, H20A(+44+71) and H20A(+149+170) directed at exons 19/20.
- Figure 11 Gel electrophoresis showing human exon 19/20 skipping using "weasels" directed at exons 19 and 20.
- 15 Figure 12 Gel electrophoresis showing exon 22 skipping using antisense molecules H22A(+125+106), H22A(+47+69), H22A(+80+101) and H22D(+13-11) directed at exon 22.
- Figure 13 Gel electrophoresis showing exon 31 skipping using antisense molecules H31D(+01-25) and H31D(+03-22); and a "cocktail" of antisense molecules directed at exon 31.
- 20 Figure 14 Gel electrophoresis showing exon 33 skipping using antisense molecules H33A(+30+56) and H33A(+64+88) directed at exon 33.
- Figure 15 Gel electrophoresis showing exon 35 skipping using antisense molecules H35A(+141+161), H35A(+116+135), and H35A(+24+43) and a "cocktail" of two antisense molecules, directed at exon 35.
- 25 Figure 16 Gel electrophoresis showing exon 36 skipping using antisense molecules H32A(+49+73) and H36A(+26+50) directed at exon 36.

- Figure 17 Gel electrophoresis showing exon 37 skipping using antisense molecules H37A(+82+105) and H37A(+134+157) directed at exon 37.
- Figure 18 Gel electrophoresis showing exon 38 skipping using antisense molecule H38A(+88+112) directed at exon 38.
- 5 Figure 19 Gel electrophoresis showing exon 40 skipping using antisense molecule H40A(-05+17) directed at exon 40.
- Figure 20 Gel electrophoresis showing exon 42 skipping using antisense molecule H42A(-04+23) directed at exon 42.
- Figure 21 Gel electrophoresis showing exon 46 skipping using antisense molecule H46A(+86+115) directed at exon 46
- 10 Figure 22 Gel electrophoresis showing exon 51, exon 52 and exon 53 skipping using various antisense molecules directed at exons 51, 52 and 53, respectively. A "cocktail" of antisense molecules is also shown directed at exon 53.

BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

15

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
1	H8A(-06+18)	GAU AGG UGG UAU CAA CAU CUG UAA
2	H8A (-03+18)	GAU AGG UGG UAU CAA CAU CUG
3	H8A(-07+18)	GAU AGG UGG UAU CAA CAU CUG UAA G
4	H8A(-06+14)	GGU GGU AUC AAC AUC UGU AA
5	H8A(-10+10)	GUA UCA ACA UCU GUA AGC AC
6	H7A(+45+67)	UGC AUG UUC CAG UCG UUG UGU GG
7	H7A(+02+26)	CAC UAU UCC AGU CAA AUA GGU CUG G
8	H7D(+15-10)	AUU UAC CAA CCU UCA GGA UCG AGU A
9	H7A(-18+03)	GGC CUA AAA CAC AUA CAC AUA
10	C6A(-10+10)	CAU UUU UGA CCU ACA UGU GG
11	C6A(-14+06)	UUU GAC CUA CAU GUG GAA AG
12	C6A(-14+12)	UAC AUU UUU GAC CUA CAU GUG GAA AG
13	C6A(-13+09)	AUU UUU GAC CUA CAU GGG AAA G
14	CH6A(+69+91)	UAC GAG UUG AUU GUC GGA CCC AG
15	C6D(+12-13)	GUG GUC UCC UUA CCU AUG ACU GUG G
16	C6D(+06-11)	GGU CUC CUU ACC UAU GA
17	H6D(+04-21)	UGU CUC AGU AAU CUU CUU ACC UAU
18	H6D(+18-04)	UCU UAC CUA UGA CUA UGG AUG AGA

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
19	H4A(+13+32)	GCA UGA ACU CUU GUG GAU CC
20	H4D(+04-16)	CCA GGG UAC UAC UUA CAU UA
21	H4D(-24-44)	AUC GUG UGU CAC AGC AUC CAG
22	H4A(+11+40)	UGU UCA GGG CAU GAA CUC UUG UGG AUC CUU
23	H3A(+30+60)	UAG GAG GCG CCU CCC AUC CUG UAG GUC ACU G
24	H3A(+35+65)	AGG UCU AGG AGG CGC CUC CCA UCC UGU AGG U
25	H3A(+30+54)	GCG CCU CCC AUC CUG UAG GUC ACU G
26	H3D(+46-21)	CUU CGA GGA GGU CUA GGA GGC GCC UC
27	H3A(+30+50)	CUC CCA UCC UGU AGG UCA CUG
28	H3D(+19-03)	UAC CAG UUU UUG CCC UGU CAG G
29	H3A(-06+20)	UCA AUA UGC UGC UUC CCA AAC UGA AA
30	H3A(+37+61)	CUA GGA GGC GCC UCC CAU CCU GUA G
31	H5A(+20+50)	UUA UGA UUU CCA UCU ACG AUG UCA GUA CUU C
32	H5D(+25-05)	CUU ACC UGC CAG UGG AGG AUU AUA UUC CAA A
33	H5D(+10-15)	CAU CAG GAU UCU UAC CUG CCA GUG G
34	H5A(+10+34)	CGA UGU CAG UAC UUC CAA UAU UCA C
35	H5D(-04-21)	ACC AUU CAU CAG GAU UCU
36	H5D(+16-02)	ACC UGC CAG UGG AGG AUU
37	H5A(-07+20)	CCA AUA UUC ACU AAA UCA ACC UGU UAA
38	H5D(+18-12)	CAG GAU UGU UAC CUG CCA GUG GAG GAU UAU
39	H5A(+05+35)	ACG AUG UCA GUA CUU CCA AUA UUC ACU AAA U
40	H5A(+15+45)	AUU UCC AUC UAC GAU GUC AGU ACU UCC AAU A
41	H10A(-05+16)	CAG GAG CUU CCA AAU GCU GCA
42	H10A(-05+24)	CUU GUC UUC AGG AGC UUC CAA AUG CUG CA
43	H10A(+98+119)	UCC UCA GCA GAA AGA AGC CAC G
44	H10A(+130+149)	UUA GAA AUC UCU CCU UGU GC
45	H10A(-33-14)	UAA AUU GGG UGU UAC ACA AU
46	H11D(+26+49)	CCC UGA GGC AUU CCC AUC UUG AAU
47	H11D(+11-09)	AGG ACU UAC UUG CUU UGU UU
48	H11A(+118+140)	CUU GAA UUU AGG AGA UUC AUC UG
49	H11A(+75+97)	CAU CUU CUG AUA AUU UUC CUG UU
50	H12A(+52+75)	UCU UCU GUU UUU GUU AGC CAG UCA
51	H12A(-10+10)	UCU AUG UAA ACU GAA AAU UU

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
52	H12A(+11+30)	UUC UGG AGA UCC AUU AAA AC
53	H13A(+77+100)	CAG CAG UUG CGU GAU CUC CAC UAG
54	H13A(+55+75)	UUC AUC AAC UAC CAC CAC CAU
55	H13D(+06-19)	CUA AGC AAA AUA AUC UGA CCU UAA G
56	H14A(+37+64)	CUU GUA AAA GAA CCC AGC GGU CUU CUG U
57	H14A(+14+35)	CAU CUA CAG AUG UUU GCC CAU C
58	H14A(+51+73)	GAA GGA UGU CUU GUA AAA GAA CC
59	H14D(-02+18)	ACC UGU UCU UCA GUA AGA CG
60	H14D(+14-10)	CAU GAC ACA CCU GUU CUU CAG UAA
61	H14A(+61+80)	CAU UUG AGA AGG AUG UCU UG
62	H14A(-12+12)	AUC UCC CAA UAC CUG GAG AAG AGA
63	H15A(-12+19)	GCC AUG CAC UAA AAA GGC ACU GCA AGA CAU U
64	H15A(+48+71)	UCU UUA AAG CCA GUU GUG UGA AUC
65	H15A(+08+28)	UUU CUG AAA GCC AUG CAC UAA
66	H15D(+17-08)	GUA CAU ACG GCC AGU UUU UGA AGA C
67	H16A(-12+19)	CUA GAU CCG CUU UUA AAA CCU GUU AAA ACA A
68	H16A(-06+25)	UCU UUU CUA GAU CCG CUU UUA AAA CCU GUU A
69	H16A(-06+19)	CUA GAU CCG CUU UUA AAA CCU GUU A
70	H16A(+87+109)	CCG UCU UCU GGG UCA CUG ACU UA
71	H16A(-07+19)	CUA GAU CCG CUU UUA AAA CCU GUU AA
72	H16A(-07+13)	CCG CUU UUA AAA CCU GUU AA
73	H16A(+12+37)	UGG AUU GCU UUU UCU UUU CUA GAU CC
74	H16A(+92+116)	CAU GCU UCC GUC UUC UGG GUC ACU G
75	H16A(+45+67)	G AUC UUG UUU GAG UGA AUA CAG U
76	H16A(+105+126)	GUU AUC CAG CCA UGC UUC CGU C
77	H16D(+05-20)	UGA UAA UUG GUA UCA CUA ACC UGU G
78	H16D(+12-11)	GUA UCA CUA ACC UGU GCU GUA C
79	H19A(+35+53)	CUG CUG GCA UCU UGC AGU U
80	H19A(+35+65)	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U
81	H20A(+44+71)	CUG GCA GAA UUC GAU CCA CCG GCU GUU C
82	H20A(+147+168)	CAG CAG UAG UUG UCA UCU GCU C
83	H20A(+185+203)	UGA UGG GGU GGU GGG UUG G
84	H20A(-08+17)	AUC UGC AUU AAC ACC CUC UAG AAA G
85	H20A(+30+53)	CCG GCU GUU CAG UUG UUC UGA GGC
86	H20A(-11+17)	AUC UGC AUU AAC ACC CUC UAG AAA GAA A
87	H20D(+08-20)	GAA GGA GAA GAG AUU CUU ACC UUA CAA A
88	H20A(+44+63)	AUU CGA UCC ACC GGC UGU UC

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
89	H20A(+149+168)	CAG CAG UAG UUG UCA UCU GC
90	H21A(-06+16)	GCC GGU UGA CUU CAU CCU GUG C
91	H21A(+85+106)	CUG CAU CCA GGA ACA UGG GUC C
92	H21A(+85+108)	GUC UGC AUC CAG GAA CAU GGG UC
93	H21A(+08+31)	GUU GAA GAU CUG AUA GCC GGU UGA
94	H21D(+18-07)	UAC UUA CUG UCU GUA GCU CUU UCU
95	H22A(+22+45)	CAC UCA UGG UCU CCU GAU AGC GCA
96	H22A(+125+106)	CUG CAA UUC CCC GAG UCU CUG C
97	H22A(+47+69)	ACU GCU GGA CCC AUG UCC UGA UG
98	H22A(+80+101)	CUA AGU UGA GGU AUG GAG AGU
99	H22D(+13-11)	UAU UCA CAG ACC UGC AAU UCC CC
100	H23A(+34+59)	ACA GUG GUG CUG AGA UAG UAU AGG CC
101	H23A(+18+39)	UAG GCC ACU UUG UUG CUC UUG C
102	H23A(+72+90)	UUC AGA GGG CGC UUU CUU C
103	H24A(+48+70)	GGG CAG GCC AUU CCU CCU UCA GA
104	H24A(-02+22)	UCU UCA GGG UUU GUA UGU GAU UCU
105	H25A(+9+36)	CUG GGC UGA AUU GUC UGA AUA UCA CUG
106	H25A(+131+156)	CUG UUG GCA CAU GUG AUC CCA CUG AG
107	H25D(+16-08)	GUC UAU ACC UGU UGG CAC AUG UGA
108	H26A(+132+156)	UGC UUU CUG UAA UUC AUC UGG AGU U
109	H26A(-07+19)	CCU CCU UUC UGG CAU AGA CCU UCC AC
110	H26A(+68+92)	UGU GUC AUC CAU UCG UGC AUC UCU G
111	H27A(+82+106)	UUA AGG CCU CUU GUG CUA CAG GUG G
112	H27A(-4+19)	GGG GCU CUU CUU UAG CUC UCU GA
113	H27D(+19-03)	GAC UUC CAA AGU CUU GCA UUU C
114	H28A(-05+19)	GCC AAC AUG CCC AAA CUU CCU AAG
115	H28A(+99+124)	CAG AGA UUU CCU CAG CUC CGC CAG GA
116	H28D(+16-05)	CUU ACA UCU AGC ACC UCA GAG
117	H29A(+57+81)	UCC GCC AUC UGU UAG GGU CUG UGC C
118	H29A(+18+42)	AUU UGG GUU AUC CUC UGA AUG UCG C
119	H29D(+17-05)	CAU ACC UCU UCA UGU AGU UCC C
120	H30A(+122+147)	CAU UUG AGC UGC GUC CAC CUU GUC UG
121	H30A(+25+50)	UCC UGG GCA GAC UGG AUG CUC UGU UC
122	H30D(+19-04)	UUG CCU GGG CUU CCU GAG GCA UU
123	H31D(+06-18)	UUC UGA AAU AAC AUA UAC CUG UGC
124	H31D(+03-22)	UAG UUU CUG AAA UAA CAU AUA CCU G
125	H31A(+05+25)	GAC UUG UCA AAU CAG AUU GGA
126	H31D(+04-20)	GUU UCU GAA AUA ACA UAU ACC UGU
127	H32D(+04-16)	CAC CAG AAA UAC AUA CCA CA
128	H32A(+151+170)	CAA UGA UUU AGC UGU GAC UG
129	H32A(+10+32)	CGA AAC UUC AUG GAG ACA UCU UG

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
130	H32A(+49+73)	CUU GUA GAC GCU GCU CAA AAU UGG C
131	H33D(+09-11)	CAU GCA CAC ACC UUU GCU CC
132	H33A(+53+76)	UCU GUA CAA UCU GAC GUC CAG UCU
133	H33A(+30+56)	GUC UUU AUC ACC AUU UCC ACU UCA GAC
134	H33A(+64+88)	CCG UCU GCU UUU UCU GUA CAA UCU G
135	H34A(+83+104)	UCC AUA UCU GUA GCU GCC AGC C
136	H34A(+143+165)	CCA GGC AAC UUC AGA AUC CAA AU
137	H34A(-20+10)	UUU CUG UUA CCU GAA AAG AAU UAU AAU GAA
138	H34A(+46+70)	CAU UCA UUU CCU UUC GCA UCU UAC G
139	H34A(+95+120)	UGA UCU CUU UGU CAA UUC CAU AUC UG
140	H34D(+10-20)	UUC AGU GAU AUA GGU UUU ACC UUU CCC CAG
141	H34A(+72+96)	CUG UAG CUG CCA GCC AUU CUG UCA AG
142	H35A(+141+161)	UCU UCU GCU CGG GAG GUG ACA
143	H35A(+116+135)	CCA GUU ACU AUU CAG AAG AC
144	H35A(+24+43)	UCU UCA GGU GCA CCU UCU GU
145	H36A(+26+50)	UGU GAU GUG GUC CAC AUU CUG GUC A
146	H36A(-02+18)	CCA UGU GUU UCU GGU AUU CC
147	H37A(+26+50)	CGU GUA GAG UCC ACC UUU GGG CGU A
148	H37A(+82+105)	UAC UAA UUU CCU GCA GUG GUC ACC
149	H37A(+134+157)	UUC UGU GUG AAA UGG CUG CAA AUC
150	H38A(-01+19)	CCU UCA AAG GAA UGG AGG CC
151	H38A(+59+83)	UGC UGA AUU UCA GCC UCC AGU GGU U
152	H38A(+88+112)	UGA AGU CUU CCU CUU UCA GAU UCA C
153	H39A(+62+85)	CUG GCU UUC UCU CAU CUG UGA UUC
154	H39A(+39+58)	GUU GUA AGU UGU CUC CUC UU
155	H39A(+102+121)	UUG UCU GUA ACA GCU GCU GU
156	H39D(+10-10)	GCU CUA AUA CCU UGA GAG CA
157	H40A(-05+17)	CUU UGA GAC CUC AAA UCC UGU U
158	H40A(+129+153)	CUU UAU UUU CCU UUC AUC UCU GGG C
159	H42A(-04+23)	AUC GUU UCU UCA CGG ACA GUG UGC UGG
160	H42A(+86+109)	GGG CUU GUG AGA CAU GAG UGA UUU
161	H42D(+19-02)	A CCU UCA GAG GAC UCC UCU UGC
162	H43D(+10-15)	UAU GUG UUA CCU ACC CUU GUC GGU C
163	H43A(+101+120)	GGA GAG AGC UUC CUG UAG CU
164	H43A(+78+100)	UCA CCC UUU CCA CAG GCG UUG CA
165	H44A(+85+104)	UUU GUG UCU UUC UGA GAA AC
166	H44D(+10-10)	AAA GAC UUA CCU UAA GAU AC
167	H44A(-06+14)	AUC UGU CAA AUC GCC UGC AG
168	H46D(+16-04)	UUA CCU UGA CUU GCU CAA GC

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
169	H46A(+90+109)	UCC AGG UUC AAG UGG GAU AC
170	H47A(+76+100)	GCU CUU CUG GGC UUA UGG GAG CAC U
171	H47D(+25-02)	ACC UUU AUC CAC UGG AGA UUU GUC UGC
172	H47A(-9+12)	UUC CAC CAG UAA CUG AAA CAG
173	H50A(+02+30)	CCA CUC AGA GCU CAG AUC UUC UAA CUU CC
174	H50A(+07+33)	CUU CCA CUC AGA GCU CAG AUC UUC UAA
175	H50D(+07-18)	GGG AUC CAG UAU ACU UAC AGG CUC C
176	H51A(-01+25)	ACC AGA GUA ACA GUC UGA GUA GGA GC
177	H51D(+16-07)	CUC AUA CCU UCU GCU UGA UGA UC
178	H51A(+111 +134)	UUC UGU CCA AGC CCG GUU GAA AUC
179	H51A(+61+90)	ACA UCA AGG AAG AUG GCA UUU CUA GUU UGG
180	H51A(+66+90)	ACA UCA AGG AAG AUG GCA UUU CUA G
181	H51A(+66+95)	CUC CAA CAU CAA GGA AGA UGG CAU UUC UAG
182	H51D(+08-17)	AUC AUU UUU UCU CAU ACC UUC UGC U
183	H51A/D(+08-17) & (-15+)	AUC AUU UUU UCU CAU ACC UUC UGC UAG GAG CUA AAA
184	H51A(+175+195)	CAC CCA CCA UCA CCC UCU GUG
185	H51A(+199+220)	AUC AUC UCG UUG AUA UCC UCA A
186	H52A(-07+14)	UCC UGC AUU GUU GCC UGU AAG
187	H52A(+12+41)	UCC AAC UGG GGA CGC CUC UGU UCC AAA UCC
188	H52A(+17+37)	ACU GGG GAC GCC UCU GUU CCA
189	H52A(+93+112)	CCG UAA UGA UUG UUC UAG CC
190	H52D(+05-15)	UGU UAA AAA ACU UAC UUC GA
191	H53A(+45+69)	CAU UCA ACU GUU GCC UCC GGU UCU G
192	H53A(+39+62)	CUG UUG CCU CCG GUU CUG AAG GUG
193	H53A(+39+69)	CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G
194	H53D(+14-07)	UAC UAA CCU UGG UUU CUG UGA
195	H53A(+23+47)	CUG AAG GUG UUC UUG UAC UUC AUC C
196	H53A(+150+176)	UGU AUA GGG ACC CUC CUU CCA UGA CUC
197	H53D(+20-05)	CUA ACC UUG GUU UCU GUG AUU UUC U
198	H53D(+09-18)	GGU AUC UUU GAU ACU AAC CUU GGU UUC
199	H53A(-12+10)	AUU CUU UCA ACU AGA AUA AAA G
200	H53A(-07+18)	GAU UCU GAA UUC UUU CAA CUA GAA U
201	H53A(+07+26)	AUC CCA CUG AUU CUG AAU UC
202	H53A(+124+145)	UUG GCU CUG GCC UGU CCU AAG A
203	H46A(+86+115)	CUC UUU UCC AGG UUC AAG UGG GAU ACU AGC

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
204	H46A(+107+137)	CAA GCU UUU CUU UUA GUU GCU GCU CUU UUC C
205	H46A(-10+20)	UAU UCU UUU GUU CUU CUA GCC UGG AGA AAG
206	H46A(+50+77)	CUG CUU CCU CCA ACC AUA AAA CAA AUU C
207	H45A(-06+20)	CCA AUG CCA UCC UGG AGU UCC UGU AA
208	H45A(+91 +110)	UCC UGU AGA AUA CUG GCA UC
209	H45A(+125+151)	UGC AGA CCU CCU GCC ACC GCA GAU UCA
210	H45D(+16 -04)	CUA CCU CUU UUU UCU GUC UG
211	H45A(+71+90)	UGU UUU UGA GGA UUG CUG AA

Table 1A: Description of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA. Since these 2'-O-methyl antisense oligonucleotides are more RNA-like, U represents uracil. With other antisense chemistries such as peptide nucleic acids or morpholinos, these U bases may be shown as "T".

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5'-3')
81	H20A(+44+71)	CUG GCA GAA UUC GAU CCA CCG GCU
82	H20A(+147+168)	GUU C CAG CAG UAG UUG UCA UCU GCU C
80	H19A(+35+65)	GCC UGA GCU GAU CUG CUG GCA UCU
81	H20A(+44+71)	UGC
82	H20A(+147+168)	AGU U CUG GCA GAA UUC GAU CCA CCG GCU GUU C CAG CAG UAG UUG UCA UCU GCU C
194	H53D(+14-07)	UAC UAA CCU UGG UUU CUG UGA
195	H53A(+23+47)	CUG AAG GUG UUC UUG UAC UUC AUC C
196	H53A(+150+175)	UGU AUA GGG ACC CUC CUU CCA UGA CUC

Table 1B: Description of a cocktail of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA.

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5'-3')
81	H20A(+44+71)-	CUG GCA GAA UUC GAU CCA CCG GCU GUU C-
82	H20A(+147+168)	CAG CAG UAG UUG UCA UCU GCU C

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5'-3')
80	H19A(+35+65)-	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U
88	H20A(+44+63)-	-AUU CGA UCC ACC GGC UGU UC-
79	H20A(+149+168)	CUG CUG GCA UCU UGC AGU U
80	H19A(+35+65)-	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U
88	H20A(+44+63)	-AUU CGA UCC ACC GGC UGU UC-
80	H19A(+35+65)-	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U
79	H20A(+149+168)	-CUG CUG GCA UCU UGC AGU U
138	H34A(+46+70)-	CAU UCA UUU CCU UUC GCA UCU UAC G-
139	H34A(+94+120)	UGA UCU CUU UGU CAA UUC CAU AUC UG
124	H31D(+03-22)-	UAG UUU CUG AAA UAA CAU AUA CCU G-
144	H35A(+24+43)	UU- UCU UCA GGU GCA CCU UCU GU
195	H53A(+23+47)-	CUG AAG GUG UUC UUG UAC UUC AUC C-
196	H53A(+150+175)-	UGU AUA GGG ACC CUC CUU CCA UGA CUC-
194	H53D(+14-07)	AA- UAC UAA CCU UGG UUU CUG UGA
- 212	Aimed at exons 19/20/20	CAG CAG UAG UUG UCA UCU GCU CAA CUG GCA GAA UUC GAU CCA CCG GCU GUU CAA GCC UGA GCU GAU CUG CUC GCA UCU UGC AGU

Table 1C: Description of a "weasel" of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA.

DETAILED DESCRIPTION OF THE INVENTION

5 General

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variation and modifications. The invention also includes all of the steps, features, compositions and compounds referred to

or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only.

- 5 Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

Sequence identity numbers (SEQ ID NO:) containing nucleotide and amino acid sequence information included in this specification are collected at the end of the description and have been prepared using the programme PatentIn Version 3.0. Each
 10 nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc.). The length, type of sequence and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification
 15 are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (e.g. <400>1, <400>2, etc.).

An antisense molecules nomenclature system was proposed and published to distinguish between the different antisense molecules (see Mann *et al.*, (2002) J Gen Med 4, 644-654). This nomenclature became especially relevant when testing several
 20 slightly different antisense molecules, all directed at the same target region, as shown below:

H # A/D (x : y).

The first letter designates the species (e.g. H: human, M: murine, C: canine) "*#*" designates target dystrophin exon number.

- 25 "A/D" indicates acceptor or donor splice site at the beginning and end of the exon, respectively.

(x y) represents the annealing coordinates where "-" or "+" indicate intronic or exonic sequences respectively. As an example, A(-6+18) would indicate the last 6 bases of the intron preceding the target exon and the first 18 bases of the target exon. The closest

splice site would be the acceptor so these coordinates would be preceded with an "A".

Describing annealing coordinates at the donor splice site could be D(+2-18) where the last 2 exonic bases and the first 18 intronic bases correspond to the annealing site of the antisense molecule. Entirely exonic annealing coordinates that would be represented by

5 A(+65+85), that is the site between the 65th and 85th nucleotide from the start of that exon.

The entire disclosures of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference. No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working in the field to which this invention relates.

As used necessarily herein the term "derived" and "derived from" shall be taken to indicate that a specific integer may be obtained from a particular source *albeit* not directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

DESCRIPTION OF THE PREFERRED EMBODIMENT

When antisense molecule(s) are targeted to nucleotide sequences involved in splicing in exons within pre-mRNA sequences, normal splicing of the exon may be inhibited causing the splicing machinery to by-pass the entire mutated exon from the mature mRNA. The concept of antisense oligonucleotide induced exon skipping is shown in Figure 2. In many genes, deletion of an entire exon would lead to the production of a non-functional protein through the loss of important functional domains or the disruption of

the reading frame. In some proteins, however, it is possible to shorten the protein by deleting one or more exons, without disrupting the reading frame, from within the protein without seriously altering the biological activity of the protein. Typically, such proteins have a structural role and or possess functional domains at their ends. The present
5 invention describes antisense molecules capable of binding to specified dystrophin pre-mRNA targets and re-directing processing of that gene.

Antisense Molecules

According to a first aspect of the invention, there is provided antisense molecules capable of binding to a selected target to induce exon skipping. To induce exon
10 skipping in exons of the Dystrophin gene transcript, the antisense molecules are preferably selected from the group of compounds shown in Table 1A. There is also provided a combination or "cocktail" of two or more antisense oligonucleotides capable of binding to a selected target to induce exon skipping. To induce exon skipping in exons of the
Dystrophin gene transcript, the antisense molecules in a "cocktail" are preferably selected
15 from the group of compounds shown in Table 1B. Alternatively, exon skipping may be induced by antisense oligonucleotides joined together "weasels" preferably selected from the group of compounds shown in Table 1C.

Designing antisense molecules to completely mask consensus splice sites may not necessarily generate any skipping of the targeted exon. Furthermore, the inventors
20 have discovered that size or length of the antisense oligonucleotide itself is not always a primary factor when designing antisense molecules. With some targets such as exon 19, antisense oligonucleotides as short as 12 bases were able to induce exon skipping, *albeit* not as efficiently as longer (20-31 bases) oligonucleotides. In some other targets, such as murine dystrophin exon 23, antisense oligonucleotides only 17 residues long were able to
25 induce more efficient skipping than another overlapping compound of 25 nucleotides.

The inventors have also discovered that there does not appear to be any standard motif that can be blocked or masked by antisense molecules to redirect splicing. In some exons, such as mouse dystrophin exon 23, the donor splice site was the most

amenable to target to re-direct skipping of that exon. It should be noted that designing and testing a series of exon 23 specific antisense molecules to anneal to overlapping regions of the donor splice site showed considerable variation in the efficacy of induced exon skipping. As reported in Mann *et al.*, (2002) there was a significant variation in the efficiency of bypassing the nonsense mutation depending upon antisense oligonucleotide annealing ("Improved antisense oligonucleotide induced exon skipping in the *mdx* mouse model of muscular dystrophy". J Gen Med 4: 644-654). Targeting the acceptor site of exon 23 or several internal domains was not found to induce any consistent exon 23 skipping.

10 In other exons targeted for removal, masking the donor splice site did not induce any exon skipping. However, by directing antisense molecules to the acceptor splice site (human exon 8 as discussed below), strong and sustained exon skipping was induced. It should be noted that removal of human exon 8 was tightly linked with the co-removal of exon 9. There is no strong sequence homology between the exon 8 antisense oligonucleotides and corresponding regions of exon 9 so it does not appear to be a matter of cross reaction. Rather the splicing of these two exons is inextricably linked. This is not an isolated instance as the same effect is observed in canine cells where targeting exon 8 for removal also resulted in the skipping of exon 9. Targeting exon 23 for removal in the mouse dystrophin pre-mRNA also results in the frequent removal of exon 22 as well. This effect occurs in a dose dependent manner and also indicates close coordinated processing of 2 adjacent exons.

25 In other targeted exons, antisense molecules directed at the donor or acceptor splice sites did not induce exon skipping while annealing antisense molecules to intra-exonic regions (*i.e.* exon splicing enhancers within human dystrophin exon 6) was most efficient at inducing exon skipping. Some exons, both mouse and human exon 19 for example, are readily skipped by targeting antisense molecules to a variety of motifs. That is, targeted exon skipping is induced after using antisense oligonucleotides to mask donor and acceptor splice sites or exon splicing enhancers.

To identify and select antisense oligonucleotides suitable for use in the modulation of exon skipping, a nucleic acid sequence whose function is to be modulated must first be identified. This may be, for example, a gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (*i.e.* splice donor sites, splice acceptor sites, or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

Preferably, the present invention aims to provide antisense molecules capable of binding to a selected target in the dystrophin pre-mRNA to induce efficient and consistent exon skipping. Duchenne muscular dystrophy arises from mutations that preclude the synthesis of a functional dystrophin gene product. These Duchenne muscular dystrophy gene defects are typically nonsense mutations or genomic rearrangements such as deletions, duplications or micro-deletions or insertions that disrupt the reading frame. As the human dystrophin gene is a large and complex gene with the 79 exons being spliced together to generate a mature mRNA with an open reading frame of approximately 11,000 bases, there are many positions where these mutations can occur. Consequently, a comprehensive antisense oligonucleotide based therapy to address many of the different disease-causing mutations in the dystrophin gene will require that many exons can be targeted for removal during the splicing process.

Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (*i.e.* splice donor sites, splice acceptor sites or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridisable" and "complementary" are terms which are used to indicate a sufficient degree of

complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense molecule need not be 100% complementary to that of its target sequence to be specifically hybridisable. An antisense molecule is specifically hybridisable when

5 binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the

10 case of *in vitro* assays, under conditions in which the assays are performed.

While the above method may be used to select antisense molecules capable of deleting any exon from within a protein that is capable of being shortened without affecting its biological function, the exon deletion should not lead to a reading frame shift in the shortened transcribed mRNA. Thus, if in a linear sequence of three exons the end of

15 the first exon encodes two of three nucleotides in a codon and the next exon is deleted then the third exon in the linear sequence must start with a single nucleotide that is capable of completing the nucleotide triplet for a codon. If the third exon does not commence with a single nucleotide there will be a reading frame shift that would lead to the generation of truncated or a non-functional protein.

20 It will be appreciated that the codon arrangements at the end of exons in structural proteins may not always break at the end of a codon, consequently there may be a need to delete more than one exon from the pre-mRNA to ensure in-frame reading of the mRNA. In such circumstances, a plurality of antisense oligonucleotides may need to be selected by the method of the invention wherein each is directed to a different region

25 responsible for inducing splicing in the exons that are to be deleted.

The length of an antisense molecule may vary so long as it is capable of binding selectively to the intended location within the pre-mRNA molecule. The length of such sequences can be determined in accordance with selection procedures described herein. Generally, the antisense molecule will be from about 10 nucleotides in length up to

about 50 nucleotides in length. It will be appreciated however that any length of nucleotides within this range may be used in the method. Preferably, the length of the antisense molecule is between 17 to 30 nucleotides in length.

In order to determine which exons can be connected in a dystrophin gene, reference should be made to an exon boundary map. Connection of one exon with another is based on the exons possessing the same number at the 3' border as is present at the 5' border of the exon to which it is being connected. Therefore, if exon 7 were deleted, exon 6 must connect to either exons 12 or 18 to maintain the reading frame. Thus, antisense oligonucleotides would need to be selected which redirected splicing for exons 7 to 11 in the first instance or exons 7 to 17 in the second instance. Another and somewhat simpler approach to restore the reading frame around an exon 7 deletion would be to remove the two flanking exons. Induction of exons 6 and 8 skipping should result in an in-frame transcript with the splicing of exons 5 to 9. In practise however, targeting exon 8 for removal from the pre-mRNA results in the co-removal of exon 9 so the resultant transcript would have exon 5 joined to exon 10. The inclusion or exclusion of exon 9 does not alter the reading frame. Once the antisense molecules to be tested have been identified, they are prepared according to standard techniques known in the art. The most common method for producing antisense molecules is the methylation of the 2' hydroxyribose position and the incorporation of a phosphorothioate backbone produces molecules that superficially resemble RNA but that are much more resistant to nuclease degradation.

To avoid degradation of pre-mRNA during duplex formation with the antisense molecules, the antisense molecules used in the method may be adapted to minimise or prevent cleavage by endogenous RNase H. This property is highly preferred as the treatment of the RNA with the unmethylated oligonucleotides either intracellularly or in crude extracts that contain RNase H leads to degradation of the pre-mRNA: antisense oligonucleotide duplexes. Any form of modified antisense molecules that is capable of bypassing or not inducing such degradation may be used in the present method. An example of antisense molecules which when duplexed with RNA are not cleaved by cellular RNase H is 2'-O-methyl derivatives. 2'-O-methyl-oligoribonucleotides are very stable in a cellular

environment and in animal tissues, and their duplexes with RNA have higher T_m values than their ribo- or deoxyribo- counterparts.

Antisense molecules that do not activate RNase H can be made in accordance with known techniques (*see, e.g.*, U.S. Pat. 5,149,797). Such antisense

5 molecules, which may be deoxyribonucleotide or ribonucleotide sequences, simply contain any structural modification which sterically hinders or prevents binding of RNase H to a duplex molecule containing the oligonucleotide as one member thereof, which structural modification does not substantially hinder or disrupt duplex formation. Because the

10 portions of the oligonucleotide involved in duplex formation are substantially different from those portions involved in RNase H binding thereto, numerous antisense molecules that do not activate RNase H are available. For example, such antisense molecules may be oligonucleotides wherein at least one, or all, of the inter-nucleotide bridging phosphate

15 residues are modified phosphates, such as methyl phosphonates, methyl phosphorothioates, phosphoromorpholidates, phosphoropiperazidates and phosphoramidates. For example, every other one of the internucleotide bridging phosphate residues may be modified as described. In another non-limiting example, such antisense molecules are molecules wherein at least one, or all, of the nucleotides contain a 2' lower alkyl moiety (*e.g.*, C_1 - C_4 , linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl, and isopropyl). For example, every other one of the nucleotides may

20 be modified as described.

While antisense oligonucleotides are a preferred form of the antisense molecules, the present invention comprehends other oligomeric antisense molecules, including but not limited to oligonucleotide mimetics such as are described below.

Specific examples of preferred antisense compounds useful in this invention

25 include oligonucleotides containing modified backbones or non-natural inter-nucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes

referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their inter-nucleoside backbone can also be considered to be oligonucleosides.

In other preferred oligonucleotide mimetics, both the sugar and the inter-nucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. Certain nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, *e.g.*, hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, *e.g.*, dodecandiol or undecyl residues, a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds that are chimeric compounds.

- 5 "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense molecules, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the increased resistance to
- 10 nuclease degradation, increased cellular uptake, and an additional region for increased binding affinity for the target nucleic acid.

Methods of Manufacturing Antisense Molecules

- The antisense molecules used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase
- 15 synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066.

- Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare
- 20 oligonucleotides such as the phosphorothioates ~ and alkylated derivatives. In one such automated embodiment, diethyl-phosphoramidites are used as starting materials and may be synthesized as described by Beaucage, *et al.*, (1981) *Tetrahedron Letters*, 22:1859-1862.

- The antisense molecules of the invention are synthesised *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed
- 25 to direct the *in vivo* synthesis of antisense molecules. The molecules of the invention may also be mixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor

targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption.

Therapeutic Agents

The present invention also can be used as a prophylactic or therapeutic,
5 which may be utilised for the purpose of treatment of a genetic disease.

Accordingly, in one embodiment the present invention provides antisense molecules that bind to a selected target in the dystrophin pre-mRNA to induce efficient and consistent exon skipping described herein in a therapeutically effective amount admixed with a pharmaceutically acceptable carrier, diluent, or excipient.

10 The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset and the like, when administered to a patient. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as
15 water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Martin, *Remington's Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

20 In a more specific form of the invention there are provided pharmaceutical compositions comprising therapeutically effective amounts of an antisense molecule together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength and additives such as detergents
25 and solubilizing agents (*e.g.*, Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol). The material may be incorporated into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into

liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g., Martin, Remington's Pharmaceutical Sciences*, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 that are herein

5 incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilised form.

It will be appreciated that pharmaceutical compositions provided according to the present invention may be administered by any means known in the art. Preferably, the pharmaceutical compositions for administration are administered by injection, orally, or
10 by the pulmonary, or nasal route. The antisense molecules are more preferably delivered by intravenous, intra-arterial, intraperitoneal, intramuscular, or subcutaneous routes of administration.

Antisense molecule based therapy

Also addressed by the present invention is the use of antisense molecules of
15 the present invention, for manufacture of a medicament for modulation of a genetic disease.

The delivery of a therapeutically useful amount of antisense molecules may be achieved by methods previously published. For example, intracellular delivery of the antisense molecule may be via a composition comprising an admixture of the antisense
20 molecule and an effective amount of a block copolymer. An example of this method is described in US patent application US 20040248833.

Other methods of delivery of antisense molecules to the nucleus are described in Mann CJ *et al.*, (2001) ["*Antisense-induced exon skipping and the synthesis of dystrophin in the mdx mouse*". *Proc., Natl. Acad. Science*, 98(1) 42-47J and in Gebiski
25 *etal.*, (2003). *Human Molecular Genetics*, 12(15): 1801-1811.

A method for introducing a nucleic acid molecule into a cell by way of an expression vector either as naked DNA or complexed to lipid carriers, is described in US patent US 6,806,084.

It may be desirable to deliver the antisense molecule in a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes or liposome formulations.

5 Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. These formulations may have net cationic, anionic or neutral charge characteristics and are useful characteristics with *in vitro*, *in vivo* and *ex vivo* delivery methods. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μ m can encapsulate a substantial percentage of an aqueous buffer
10 containing large macromolecules. RNA, and DNA can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al.*, Trends Biochem. Sci., 6:77, 1981).

In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the antisense molecule of interest at
15 high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al.*, Biotechniques, 6:682, 1988).

20 The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

25 Alternatively, the antisense construct may be combined with other pharmaceutically acceptable carriers or diluents to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

The routes of administration described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and any dosage for any particular animal and condition. Multiple approaches for introducing functional new genetic material into cells, both *in vitro* and *in vivo* have been attempted (Friedmann (1989) Science, 244:1275-1280).

These approaches include integration of the gene to be expressed into modified retroviruses (Friedmann (1989) supra; Rosenberg (1991) Cancer Research 51(18), suppl.: 5074S-5079S); integration into non-retrovirus vectors (Rosenfeld, *et al.* (1992) Cell, 68:143-155; Rosenfeld, *et al.* (1991) Science, 252:431-434); or delivery of a transgene linked to a heterologous promoter-enhancer element via liposomes (Friedmann (1989), supra; Brigham, *et al.* (1989) Am. J. Med. Sci., 298:278-281; Nabel, *et al.* (1990) Science, 249:1285-1288; Hazinski, *et al.* (1991) Am. J. Resp. Cell Molec. Biol., 4:206-209; and Wang and Huang (1987) Proc. Natl. Acad. Sci. (USA), 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu (1988) J. Biol. Chem., 263:14621-14624) or the use of naked DNA, expression vectors (Nabel *et al.* (1990), supra); Wolff *et al.* (1990) Science, 247:1465-1468). Direct injection of transgenes into tissue produces only localized expression (Rosenfeld (1992) supra; Rosenfeld *et al.* (1991) supra; Brigham *et al.* (1989) supra; Nabel (1990) supra; and Hazinski *et al.* (1991) supra). The Brigham *et al.* group (Am. J. Med. Sci. (1989) 298:278-281 and Clinical Research (1991) 39 (abstract)) have reported *in vivo* transfection only of lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. An example of a review article of human gene therapy procedures is: Anderson, Science (1992) 256:808-813.

The antisense molecules of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the

compounds of the invention, pharmaceutically acceptable salts of such pro-drugs, and other bioequivalents.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: *i.e.*, salts that retain
5 the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b)
10 acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, malefic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid,
15 methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including
20 ophthalmic and to mucous membranes including rectal delivery), pulmonary, *e.g.*, by inhalation or insufflation of powders or aerosols, (including by nebulizer, intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intra-arterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, *e.g.*, intrathecal or intraventricular, administration.
25 Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of

bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

5 Kits of the Invention

The invention also provides kits for treatment of a patient with a genetic disease which kit comprises at least an antisense molecule, packaged in a suitable container, together with instructions for its use.

In a preferred embodiment, the kits will contain at least one antisense
10 molecule as shown in Table 1A, or a cocktail of antisense molecules as shown in Table 1B or a "weasel" compound as shown in Table 1C. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

Those of ordinary skill in the field should appreciate that applications of the above method has wide application for identifying antisense molecules suitable for use in
15 the treatment of many other diseases.

EXAMPLES

The following Examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these Examples in no
20 way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. The references cited herein are expressly incorporated by reference.

Methods of molecular cloning, immunology and protein chemistry, which are not explicitly described in the following examples, are reported in the literature and are known by those skilled in the art. General texts that described conventional molecular
25 biology, microbiology, and recombinant DNA techniques within the skill of the art, included, for example: Sambrook *et al*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989);

Glover ed., *DNA Cloning: A Practical Approach*, Volumes I and II, MRL Press, Ltd., Oxford, U.K. (1985); and Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. *Current Protocols in Molecular Biology*. Greene Publishing Associates/Wiley Intersciences, New York (2002).

5 DETERMINING INDUCED EXON SKIPPING IN HUMAN MUSCLE CELLS

Attempts by the inventors to develop a rational approach in antisense molecules design were not completely successful as there did not appear to be a consistent trend that could be applied to all exons. As such, the identification of the most effective and therefore most therapeutic antisense molecules compounds has been the result of
10 empirical studies.

These empirical studies involved the use of computer programs to identify motifs potentially involved in the splicing process. Other computer programs were also used to identify regions of the pre-mRNA which may not have had extensive secondary structure and therefore potential sites for annealing of antisense molecules. Neither of
15 these approaches proved completely reliable in designing antisense oligonucleotides for reliable and efficient induction of exon skipping.

Annealing sites on the human dystrophin pre-mRNA were selected for examination, initially based upon known or predicted motifs or regions involved in splicing. 2OMe antisense oligonucleotides were designed to be complementary to the
20 target sequences under investigation and were synthesised on an Expedite 8909 Nucleic Acid Synthesiser. Upon completion of synthesis, the oligonucleotides were cleaved from the support column and de-protected in ammonium hydroxide before being desalted. The quality of the oligonucleotide synthesis was monitored by the intensity of the trityl signals upon each deprotection step during the synthesis as detected in the synthesis log. The
25 concentration of the antisense oligonucleotide was estimated by measuring the absorbance of a diluted aliquot at 260nm.

Specified amounts of the antisense molecules were then tested for their ability to induce exon skipping in an *in vitro* assay, as described below.

Briefly, normal primary myoblast cultures were prepared from human muscle biopsies obtained after informed consent. The cells were propagated and allowed to differentiate into myotubes using standard culturing techniques. The cells were then transfected with the antisense oligonucleotides by delivery of the oligonucleotides to the
5 dells as cationic lipoplexes, mixtures of antisense molecules or cationic liposome preparations.

The cells were then allowed to grow for another 24 hours, after which total RNA was extracted and molecular analysis commenced. Reverse transcriptase amplification (RT-PCR) was undertaken to study the targeted regions of the dystrophin
10 pre-mRNA or induced exonic re-arrangements.

For example, in the testing of an antisense molecule for inducing exon 19 skipping the RT-PCR test scanned several exons to detect involvement of any adjacent exons. For example, when inducing skipping of exon 19, RT-PCR was carried out with primers that amplified across exons 17 and 21. Amplifications of even larger products in
15 this area (*i.e.* exons 13-26) were also carried out to ensure that there was minimal amplification bias for the shorter induced skipped transcript. Shorter or exon skipped products tend to be amplified more efficiently and may bias the estimated of the normal and induced transcript.

The sizes of the amplification reaction products were estimated on an
20 agarose gel and compared against appropriate size standards. The final confirmation of identity of these products was carried out by direct DNA sequencing to establish that the correct or expected exon junctions have been maintained.

Once efficient exon skipping had been induced with one antisense molecule, subsequent overlapping antisense molecules may be synthesized and then evaluated in the
25 assay as described above. Our definition of an efficient antisense molecule is one that induces strong and sustained exon skipping at transfection concentrations in the order of 300 nM or less.

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 8

Antisense oligonucleotides directed at exon 8 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

5 Figure 3 shows differing efficiencies of two antisense molecules directed at exon 8 acceptor splice site. H8A(-06+18) [SEQ ID NO:1], which anneals to the last 6 bases of intron 7 and the first 18 bases of exon 8, induces substantial exon 8 and 9 skipping when delivered into cells at a concentration of 20 nM. The shorter antisense molecule, H8A(-06+14) [SEQ ID NO: 4] was only able to induce exon 8 and 9 skipping at 300 nM, a
10 concentration some 15 fold higher than H8A(-06+18), which is the preferred antisense molecule.

 This data shows that some particular antisense molecules induce efficient exon skipping while another antisense molecule, which targets a near-by or overlapping region, can be much less efficient. Titration studies show one compound is able to induce
15 targeted exon skipping at 20 nM while the less efficient antisense molecules only induced exon skipping at concentrations of 300 nM and above. Therefore, we have shown that targeting of the antisense molecules to motifs involved in the splicing process plays a crucial role in the overall efficacy of that compound.

 Efficacy refers to the ability to induce consistent skipping of a target exon.
20 However, sometimes skipping of the target exons is consistently associated with a flanking exon. That is, we have found that the splicing of some exons is tightly linked. For example, in targeting exon 23 in the mouse model of muscular dystrophy with antisense molecules directed at the donor site of that exon, dystrophin transcripts missing exons 22 and 23 are frequently detected. As another example, when using an antisense molecule
25 directed to exon 8 of the human dystrophin gene, all induced transcripts are missing both exons 8 and 9. Dystrophin transcripts missing only exon 8 are not observed.

 Table 2 below discloses antisense molecule sequences that induce exon 8 (and 9) skipping.

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
1	H8A(-06+18)	5'-GAU AGG UGG UAU CAA CAU CUG UAA	Very strong to 20 nM
2	H8A (-03+18)	5'-GAU AGG UGG UAU CAA CAU CUG	Very strong skipping to 40nM
3	H8A(-07+18)	5'-GAU AGG UGG UAU CAA CAU CUG UAA G	Strong skipping to 40nM
4	H8A(-06+14)	5'-GGU GGU AUC AAC AUC UGU AA	Skipping to 300nM
5	H8A(-10+10)	5'-GUA UCA ACA UCU GUA AGC AC	Patchy/weak skipping to 100nm

Table 2

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 7

Antisense oligonucleotides directed at exon 7 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

5 described above.

Figure 4 shows the preferred antisense molecule, H7A(+45+67) [SEQ ID NO: 6], and another antisense molecule, H7A(+2+26) [SEQ ID NO: 7], inducing exon 7 skipping. Nested amplification products span exons 3 to 9. Additional products above the induced transcript missing exon 7 arise from amplification from carry-over outer primers

10 from the RT-PCR as well as heteroduplex formation.

Table 3 below discloses antisense molecule sequences for induced exon 7 skipping.

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
6	H7A(+45+67)	5' - UGC AUG UUC CAG UCG UUG UGU GG	Strong skipping to 20nM
7	H7A(+02+26)	5' - CAC UAU UCC AGU CAA AUA GGU CUG G	Weak skipping at 100nM
8	H7D(+15-10)	5' -AUU UAC CAA CCU UCA GGA UCG AGU A	Weak skipping to 300nM
9	H7A(-18+03)	5' - GGC CUA AAA CAC AUA CAC AUA	Weak skipping to 300nM

Table 3

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 6

Antisense oligonucleotides directed at exon 6 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 5 shows an example of two non-preferred antisense molecules inducing very low levels of exon 6 skipping in cultured human cells. Targeting this exon for specific removal was first undertaken during a study of the canine model using the oligonucleotides as listed in Table 4, below. Some of the human specific oligonucleotides were also evaluated, as shown in Figure 5. In this example, both antisense molecules target the donor splice site and only induced low levels of exon 6 skipping. Both H6D(+4-21) [SEQ ID NO: 17] and H6D(+18-4) [SEQ ID NO: 18] would be regarded as non-preferred antisense molecules.

One antisense oligonucleotide that induced very efficient exon 6 skipping in the canine model, C6A(+69+91) [SEQ ID NO: 14], would anneal perfectly to the corresponding region in human dystrophin exon 6. This compound was evaluated, found to be highly efficient at inducing skipping of that target exon, as shown in Figure 6 and is regarded as the preferred compound for induced exon 6 skipping. Table 4 below discloses antisense molecule sequences for induced exon 6 skipping.

SEQ ID	Antisense Oligo name	Sequence	Ability to induce skipping
10	C6A(-10+10)	5' CAU UUU UGA CCU ACA UGU GG	No skipping
11	C6A(-14+06)	5' UUU GAC CUA CAU GUG GAA AG	No skipping
12	C6A(-14+12)	5' UAC AUU UUU GAC CUA CAU GUG GAA AG	No skipping
13	C6A(-13+09)	5' AUU UUU GAC CUA CAU GGG AAA G	No skipping
14	CH6A(+69+91)	5' UAC GAG UUG AUU GUC GGA CCC AG	Strong skipping to 20 nM
15	C6D(+12-13)	5' GUG GUC UCC UUA CCU AUG ACU GUG G	Weak skipping at 300 nM
16	C6D(+06-11)	5' GGU CUC CUU ACC UAU GA	No skipping
17	H6D(+04-21)	5' UGU CUC AGU AAU CUU CUU ACC UAU	Weak skipping to 50 nM
18	H6D(+18-04)	5' UCU UAC CUA UGA CUA UGG AUG AGA	Very weak skipping to 300 nM

Table 4

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 4

Antisense oligonucleotides directed at exon 4 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 7 shows an example of a preferred antisense molecule inducing skipping of exon 4 skipping in cultured human cells. In this example, one preferred antisense compound, H4A(+13+32) [SEQ ID NO:19], which targeted a presumed exonic splicing enhancer induced efficient exon skipping at a concentration of 20 nM while other non-preferred antisense oligonucleotides failed to induce even low levels of exon 4 skipping. Another preferred antisense molecule inducing skipping of exon 4 was H4A(+111+40) [SEQ ID NO:22], which induced efficient exon skipping at a concentration of 20 nM.

Table 5 below discloses antisense molecule sequences for inducing exon 4 skipping.

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
19	H4A(+13+32)	5' GCA UGA ACU CUU GUG GAU CC	Skipping to 20 nM
22	H4A(+11+40)	5' UGU UCA GGG CAU GAA CUC UUG UGG AUC CUU	Skipping to 20 nM
20	H4D(+04-16)	5' CCA GGG UAC UAC UUA CAU UA	No skipping
21	H4D(-24-44)	5' AUC GUG UGU CAC AGC AUC CAG	No skipping

Table 5

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 3

Antisense oligonucleotides directed at exon 3 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H3A(+30+60) [SEQ ID NO:23] induced substantial exon 3 skipping when delivered into cells at a concentration of 20 nM to 600 nM. The antisense molecule, H3A(+35+65) [SEQ ID NO: 24] induced exon skipping at 300 nM.

Table 6 below discloses antisense molecule sequences that induce exon 3 skipping.

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
23	H3A(+30+60)	UAG GAG GCG CCU CCC AUC CUG UAG GUC ACU G	Moderate skipping to 20 to 600 nM
24	H3A(+35+65)	AGG UCU AGG AGG CGC CUC CCA UCC UGU AGG U	Working to 300 nM
25	H3A(+30+54)	GCG CCU CCC AUC CUG UAG GUC ACU G	Moderate 100-600 nM
26	H3D(+46-21)	CUU CGA GGA GGU CUA GGA GGC GCC UC	No skipping
27	H3A(+30+50)	CUC CCA UCC UGU AGG UCA CUG	Moderate 20-600 nM
28	H3D(+19-03)	UAC CAG UUU UUG CCC UGU CAG G	No skipping
29	H3A(-06+20)	UCA AUA UGC UGC UUCCCA AAC UGA AA	No skipping

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
30	H3A(+37+61)	CUA GGA GGC GCC UCC CAU CCU GUA G	No skipping

Table 6

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 5

Antisense oligonucleotides directed at exon 5 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

5 described above.

H5A(+20+50) [SEQ ID NO:31] induces substantial exon 5 skipping when delivered into cells at a concentration of 100 nM. Table 7 below shows other antisense molecules tested. The majority of these antisense molecules were not as effective at exon skipping as H5A(+20+50). However, H5A(+15+45) [SEQ ID NO: 40] was able to induce

10 exon 5 skipping at 300 nM.

Table 7 below discloses antisense molecule sequences that induce exon 5 skipping.

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
31	H5A(+20+50)	UUA UGA UUU CCA UCU ACG AUG UCA GUA CUU C	Working to 100 nM
32	H5D(+25-05)	CUU ACC UGC CAG UGG AGG AUU AUA UUC CAA A	No skipping
33	H5D(+10-15)	CAU CAG GAU UCU UAC CUG CCA GUG G	Inconsistent at 300 nM
34	H5A(+10+34)	CGA UGU CAG UAC UUC CAA UAU UCA C	Very weak
35	H5D(-04-21)	ACC AUU CAU CAG GAU UCU	No skipping
36	H5D(+16-02)	ACC UGC CAG UGG AGG AUU	No skipping
37	H5A(-07+20)	CCA AUA UUC ACU AAA UCA ACC UGU UAA	No skipping
38	H5D(+18-12)	CAG GAU UCU UAC CUG CCA GUG GAG GAU UAU	No skipping

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
39	H5A(+05+35)	ACG AUG UCA GUA CUU CCA AUA UUC ACU AAA U	No skipping
40	H5A(+15+45)	AUU UCC AUC UAC GAU GUC AGU ACU UCC AAU A	Working to 300 nM

Table 7

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 10

Antisense oligonucleotides directed at exon 10 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

5 described above.

H10A(-05+16) [SEQ ID NO:41] induced substantial exon 10 skipping when delivered into cells. Table 8 below shows other antisense molecules tested. The antisense molecules ability to induce exon skipping was variable. Table 8 below discloses antisense molecule sequences that induce exon 10 skipping.

10

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
41	H10A(-05+16)	CAG GAG CUU CCA AAU GCU GCA	Not tested
42	H10A(-05+24)	CUU GUC UUC AGG AGC UUC CAA AUG CUG CA	Not tested
43	H10A(+98+119)	UCC UCA GCA GAA AGA AGC CAC G	Not tested
44	H10A(+130+149)	UUA GAA AUC UCU CCU UGU GC	No skipping
45	H10A(-33-14)	UAA AUU GGG UGU UAC ACA AU	No skipping

Table 8

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 11

Antisense oligonucleotides directed at exon 11 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

15 described above.

Figure 8B shows an example of H11A(+75+97) [SEQ ID NO:49] antisense molecule inducing exon 11 skipping in cultured human cells. H11A(+75+97) induced substantial exon 11 skipping when delivered into cells at a concentration of 5 nM. Table 9

below shows other antisense molecules tested. The antisense molecules ability to induce exon skipping was observed at 100 nM.

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
46	H11D(+26+49)	CCC UGA GGC AUU CCC AUC UUG AAU	Skipping at 100 nM
47	H11D(+11-09)	AGG ACU UAC UUG CUU UGU UU	Skipping at 100 nM
48	H11A(+118+140)	CUU GAA UUU AGG AGA UUC AUC UG	Skipping at 100 nM
49	H11A(+75+97)	CAU CUU CUG AUA AUU UUC CUG UU	Skipping at 100 nM
46	H11D(+26+49)	CCC UGA GGC AUU CCC AUC UUG AAU	Skipping at 5nM

Table 9

5 ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 12

Antisense oligonucleotides directed at exon 12 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H12A(+52+75) [SEQ ID NO:50] induced substantial exon 12 skipping when delivered into cells at a concentration of 5 nM, as shown in Figure 8A. Table 10 below shows other antisense molecules tested at a concentration range of 5, 25, 50, 100, 200 and 300 nM. The antisense molecules ability to induce exon skipping was variable.

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
50	H12A(+52+75)	UCU UCU GUU UUU GUU AGC CAG UCA	Skipping at 5 nM
51	H12A(-10+10)	UCU AUG UAA ACU GAA AAU UU	Skipping at 100 nM
52	H12A(+11+30)	UUC UGG AGA UCC AUU AAA AC	No skipping

Table 10

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 13

Antisense oligonucleotides directed at exon 13 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

- 5 H13A(+77+100) [SEQ ID NO:53] induced substantial exon 13 skipping when delivered into cells at a concentration of 5 nM. Table 11 below includes two other antisense molecules tested at a concentration range of 5, 25, 50, 100, 200 and 300 nM. These other antisense molecules were unable to induce exon skipping.

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
53	H13A(+77+100)	CAG CAG UUG CGU GAU CUC CAC UAG	Skipping at 5 nM
54	H13A(+55+75)	UUC AUC AAC UAC CAC CAC CAU	No skipping
55	H13D(+06-19)	CUA AGC AAA AUA AUC UGA CCU UAA G	No skipping

10

Table 11

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 14

Antisense oligonucleotides directed at exon 14 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

- 15 H14A(+37+64) [SEQ ID NO:56] induced weak exon 14 skipping when delivered into cells at a concentration of 100 nM. Table 12 below includes other antisense molecules tested at a concentration range of 5, 25, 50, 100, 200 and 300 nM. The other antisense molecules were unable to induce exon skipping at any of the concentrations tested.

20

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
56	H14A(+37+64)	CUU GUA AAA GAA CCC AGC GGU CUU CUG U	Skipping at 100 nM

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
57	H14A(+14+35)	CAU CUA CAG AUG UUU GCC CAU C	No skipping
58	H14A(+51+73)	GAA GGA UGU CUU GUA AAA GAA CC	No skipping
59	H14D(-02+18)	ACC UGU UCU UCA GUA AGA CG	No skipping
60	H14D(+14-10)	CAU GAC ACA CCU GUU CUU CAG UAA	No skipping
61	H14A(+61 +80)	CAU UUG AGA AGG AUG UCU UG	No skipping
62	H14A(-12+12)	AUC UCC CAA UAC CUG GAG AAG AGA	No skipping

Table 12

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 15

Antisense oligonucleotides directed at exon 15 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

5 described above.

H15A(-12+19) [SEQ ID NO:63] and H15A(+48+71) [SEQ ID NO:64] induced substantial exon 15 skipping when delivered into cells at a concentration of 10 Nm, as shown in Figure 9A. Table 13 below includes other antisense molecules tested at a concentration range of 5, 25, 50, 100, 200 and 300 Nm. These other antisense molecules

10 were unable to induce exon skipping at any of the concentrations tested.

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
63	H15A(-12+19)	GCC AUG CAC UAA AAA GGC ACU GCA AGA CAU U	Skipping at 5Nm
64	H15A(+48+71)	UCU UUA AAG CCA GUU GUG UGA AUC	Skipping at 5Nm
65	H15A(+08+28)	UUU CUG AAA GCC AUG CAC UAA	No skipping
63	H15A(-12+19)	GCC AUG CAC UAA AAA GGC ACU GCA AGA CAU U	No skipping
66	H15D(+17-08)	GUA CAU ACG GCC AGU UUU UGA AGA C	No skipping

Table 13

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 16

Antisense oligonucleotides directed at exon 16 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

5 described above.

H16A(-12+19) [SEQ ID NO:67] and H16A(-06+25) [SEQ ID NO:68] induced substantial exon 16 skipping when delivered into cells at a concentration of 10 nM, as shown in Figure 9B. Table 14 below includes other antisense molecules tested. H16A(-06+19) [SEQ ID NO:69] and H16A(+87+109) [SEQ ID NO:70] were tested at a
 10 concentration range of 5, 25, 50, 100, 200 and 300 nM. These two antisense molecules were able to induce exon skipping at 25 nM and 100 nM, respectively. Additional antisense molecules were tested at 100, 200 and 300 nM and did not result in any exon skipping.

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
67	H16A(-12+19)	CUA GAU CCG CUU UUA AAA CCU GUU AAA ACA A	Skipping at 5 nM
68	H16A(-06+25)	UCU UUU CUA GAU CCG CUU UUA AAA CCU GUU A	Skipping at 5 nM
69	H16A(-06+19)	CUA GAU CCG CUU UUA AAA CCU GUU A	Skipping at 25 nM
70	H16A(+87+109)	CCG UCU UCU GGG UCA CUG ACU UA	Skipping at 100 nM

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
71	H16A(-07+19)	CUA GAU CCG CUU UUA AAA CCU GUU AA	No skipping
72	H16A(-07+13)	CCG CUU UUA AAA CCU GUU AA	No skipping
73	H16A(+12+37)	UGG AUU GCU UUU UCU UUU CUA GAU CC	No skipping
74	H16A(+92+116)	CAU GCU UCC GUC UUC UGG GUC ACU G	No skipping
75	H16A(+45+67)	G AUC UUG UUU GAG UGA AUA CAG U	No skipping
76	H16A(+105+126)	GUU AUC CAG CCA UGC UUC CGU C	No skipping
77	H16D(+05-20)	UGA UAA UUG GUA UCA CUA ACC UGU G	No skipping
78	H16D(+12-11)	GUA UCA CUA ACC UGU GCU GUA C	No skipping

Table 14

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 19

Antisense oligonucleotides directed at exon 19 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

5 described above.

H19A(+35+65) [SEQ ID NO:79] induced substantial exon 19 skipping when delivered into cells at a concentration of 10 nM. This antisense molecule also showed very strong exon skipping at concentrations of 25, 50, 100, 300 and 600 nM.

Figure 10 illustrates exon 19 and 20 skipping using a "cocktail" of antisense
 10 oligonucleotides, as tested using gel electrophoresis. It is interesting to note that it was not easy to induce exon 20 skipping using single antisense oligonucleotides H20A(+44+71) [SEQ ID NO:81] or H20A(+149+170) [SEQ ID NO:82], as illustrated in sections 2 and 3 of the gel shown in Figure 10. Whereas, a "cocktail" of antisense oligonucleotides was more efficient as can be seen in section 4 of Figure 10 using a "cocktail" of antisense
 15 oligonucleotides H20A(+44+71) and H20A(+149+170). When the cocktail was used to target exon 19, skipping was even stronger (see section 5, Figure 10).

Figure 11 illustrates gel electrophoresis results of exon 19/20 skipping using "weasels" The "weasels" were effective in skipping exons 19 and 20 at concentrations of 25, 50, 100, 300 and 600 nM. A further "weasel" sequence is shown in the last row of
 20 Table 3C. This compound should give good results.

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 20

Antisense oligonucleotides directed at exon 20 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

5 None of the antisense oligonucleotides tested induced exon 20 skipping when delivered into cells at a concentration of 10, 25, 50, 300 or 600 nM (see Table 15). Antisense molecules H20A(-11+17) [SEQ ID NO:86] and H20D(+08-20) [SEQ ID NO:87] are yet to be tested.

10 However, a combination or "cocktail" of H20A(+44+71) [SEQ ID NO: 81] and H20(+149+170) [SEQ ID NO:82] in a ratio of 1:1, exhibited very strong exon skipping at a concentration of 100 nM and 600 nM. Further, a combination of antisense molecules H19A(+35+65) [SEQ ID NO:79], H20A(+44+71) [SEQ ID NO:81] and H20A(+149+170) [SEQ ID NO:82] in a ratio of 2:1:1, induced very strong exon skipping at a concentration ranging from 10 nM to 600 nM.

15

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
81	H20A(+44+71)	CUG GCA GAA UUC GAU CCA CCG GCU GUU C	No skipping
82	H20A(+147+168)	CAG CAG UAG UUG UCA UCU GCU C	No skipping
83	H20A(+185+203)	UGA UGG GGU GGU GGG UUG G	No skipping
84	H20A(-08+17)	AUC UGC AUU AAC ACC CUC UAG AAA G	No skipping
85	H20A(+30+53)	CCG GCU GUU CAG UUG UUC UGA GGC	No skipping
86	H20A(-11+17)	AUC UGC AUU AAC ACC CUC UAG AAA GAA A	Not tested yet
87	H20D(+08-20)	GAA GGA GAA GAG AUU CUU ACC UUA CAA A	Not tested yet
81 & 82	H20A(+44+71) & H20A(+147+168)	CUG GCA GAA UUC GAU CCA CCG GCU GUU C CAG CAG UAG UUG UCA UCU GCU C	Very strong skipping
80, 81	H19A(+35+65);	GCC UGA GCU GAU CUG CUG GCA UCU	Very strong

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
& 82	H20A(+44+71); H20A(+147+168)	UGC AGU U; CUG GCA GAA UUC GAU CCA CCG GCU GUU C; CAG CAG UAG UUG UCA UCU GCU C	skipping

Table 15

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 21

Antisense oligonucleotides directed at exon 21 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

5 described above.

H21A(+85+108) [SEQ ID NO:92] and H21A(+85+106) [SEQ ID NO:91] induced exon 21 skipping when delivered into cells at a concentration of 50 nM. Table 16 below includes other antisense molecules tested at a concentration range of 5, 25, 50, 100, 200 and 300 nM. These antisense molecules showed a variable ability to induce exon

10 skipping

SEQ ID	Antisense Oligonucleotide name	Sequence	Ability to induce skipping
90	H21A(-06+16)	GCC GGU UGA CUU CAU CCU GUG C	Skips at 600 nM
91	H21A(+85+106)	CUG CAU CCA GGA ACA UGG GUC C	Skips at 50 nM
92	H21A(+85+108)	GUC UGC AUC CAG GAA CAU GGG UC	Skips at 50 nM
93	H21A(+08+31)	GUU GAA GAU CUG AUA GCC GGU UGA	Skips faintly to
94	H21D(+18-07)	UAC UUA CUG UCU GUA GCU CUU UCU	No skipping

Table 16

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 22

Antisense oligonucleotides directed at exon 22 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

15 described above.

Figure 12 illustrates differing efficiencies of two antisense molecules directed at exon 22 acceptor splice site. H22A(+125+106) [SEQ ID NO:96] and H22A(+80+101) [SEQ ID NO: 98] induce strong exon 22 skipping from 50 nM to 600 nM concentration.

- 5 H22A(+125+146) [SEQ ID NO:96] and H22A(+80+101) [SEQ ID NO:98] induced exon 22 skipping when delivered into cells at a concentration of 50 nM. Table 17 below shows other antisense molecules tested at a concentration range of 50, 100, 300 and 600 nM. These antisense molecules showed a variable ability to induce exon skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
95	H22A(+22+45)	CAC UCA UGG UCU CCU GAU AGC GCA	No skipping
96	H22A(+125+146)	CUG CAA UUC CCC GAG UCU CUG C	Skipping to 50 nM
97	H22A(+47+69)	ACU GCU GGA CCC AUG UCC UGA UG	Skipping to 300 nM
98	H22A(+80+101)	CUA AGU UGA GGU AUG GAG AGU	Skipping to 50 nM
99	H22D(+13-11)	UAU UCA CAG ACC UGC AAU UCC CC	No skipping

10 Table 17

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 23

Antisense oligonucleotides directed at exon 23 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

- 15 Table 18 below shows antisense molecules tested at a concentration range of 25, 50, 100, 300 and 600 nM. These antisense molecules showed no ability to induce exon skipping or are yet to be tested.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
100	H23A(+34+59)	ACA GUG GUG CUG AGA UAG UAU AGG CC	No skipping
101	H23A(+18+39)	UAG GCC ACU UUG UUG CUC UUG C	No Skipping
102	H23A(+72+90)	UUC AGA GGG CGC UUU CUU C	No Skipping

Table 18

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 24

Antisense oligonucleotides directed at exon 24 were prepared using similar methods as described above. Table 19 below outlines the antisense oligonucleotides

5 directed at exon 24 that are yet to be tested for their ability to induce exon 24 skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
103	H24A(+48+70)	GGG CAG GCC AUU CCU CCU UCA GA	Needs testing
104	H24A(-02+22)	UCU UCA GGG UUU GUA UGU GAU UCU	Needs testing

Table 19

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 25

Antisense oligonucleotides directed at exon 25 were prepared using similar

10 methods as described above. Table 20 below shows the antisense oligonucleotides directed at exon 25 that are yet to be tested for their ability to induce exon 25 skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
105	H25A(+9+36)	CUG GGC UGA AUU GUC UGA AUA UCA CUG	Needs testing
106	H25A(+131+156)	CUG UUG GCA CAU GUG AUC CCA CUG AG	Needs testing
107	H25D(+16-08)	GUC UAU ACC UGU UGG CAC AUG UGA	Needs testing

Table 20

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 26

Antisense oligonucleotides directed at exon 26 were prepared using similar methods as described above. Table 21 below outlines the antisense oligonucleotides directed at exon 26 that are yet to be tested for their ability to induce exon 26 skipping.

5

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
108	H26A(+132+156)	UGC UUU CUG UAA UUC AUC UGG AGU U	Needs testing
109	H26A(-07+19)	CCU CCU UUC UGG CAU AGA CCU UCC AC	Needs testing
110	H26A(+68+92)	UGU GUC AUC CAU UCG UGC AUC UCU G	Faint skipping at 600 nM

Table 21

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 27

Antisense oligonucleotides directed at exon 27 were prepared using similar methods as described above. Table 22 below outlines the antisense oligonucleotides directed at exon 27 that are yet to be tested for their ability to induce exon 27 skipping.

10

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
111	H27A(+82+106)	UUA AGG CCU CUU GUG CUA CAG GUG G	Needs testing
112	H27A(-4+19)	GGG CCU CUU CUU UAG CUC UCU GA	Faint skipping at 600 and 300 nM
113	H27D(+19-03)	GAC UUC CAA AGU CUU GCA UUU C	v. strong skipping at 600 and 300 nM

Table 22

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 28

Antisense oligonucleotides directed at exon 28 were prepared using similar methods as described above. Table 23 below outlines the antisense oligonucleotides directed at exon 28 that are yet to be tested for their ability to induce exon 28 skipping.

15

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
114	H28A(-05+19)	GCC AAC AUG CCC AAA CUU CCU AAG	v. strong skipping at 600 and 300 nM
115	H28A(+99+124)	CAG AGA UUU CCU CAG CUC CGC CAG GA	Needs testing
116	H28D(+16-05)	CUU ACA UCU AGC ACC UCA GAG	v. strong skipping at 600 and 300 nM

Table 23

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 29

Antisense oligonucleotides directed at exon 29 were prepared using similar methods as described above. Table 24 below outlines the antisense oligonucleotides directed at exon 29 that are yet to be tested for their ability to induce exon 29 skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
117	H29A(+57+81)	UCC GCC AUC UGU UAG GGU CUG UGC C	Needs testing
118	H29A(+18+42)	AUU UGG GUU AUC CUC UGA AUG UCG C	v. strong skipping at 600 and 300 nM
119	H29D(+17-05)	CAU ACC UCU UCA UGU AGU UCC C	v. strong skipping at 600 and 300 nM

Table 24

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 30

Antisense oligonucleotides directed at exon 30 were prepared using similar methods as described above. Table 25 below outlines the antisense oligonucleotides directed at exon 30 that are yet to be tested for their ability to induce exon 30 skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
120	H30A(+122+147)	CAU UUG AGC UGC GUC CAC CUU GUC UG	Needs testing
121	H30A(+25+50)	UCC UGG GCA GAC UGG AUG CUC UGU UC	Very strong skipping at 600 and 300 nM.
122	H30D(+19-04)	UUG CCU GGG CUU CCU GAG GCA UU	Very strong skipping at 600 and 300 nM.

Table 25

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 31

Antisense oligonucleotides directed at exon 31 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 13 illustrates differing efficiencies of two antisense molecules directed at exon 31 acceptor splice site and a "cocktail" of exon 31 antisense oligonucleotides at varying concentrations. H31D(+03-22) [SEQ ID NO:124] substantially induced exon 31 skipping when delivered into cells at a concentration of 20 nM. Table 26 below also includes other antisense molecules tested at a concentration of 100 and 300 nM. These antisense molecules showed a variable ability to induce exon skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
123	H31D(+06-18)	UUC UGA AAU AAC AUA UAC CUG UGC	Skipping to 300 nM
124	H31D(+03-22)	UAG UUU CUG AAA UAA CAU AUA CCU G	Skipping to 20 nM
125	H31A(+05+25)	GAC UUG UCA AAU CAG AUU GGA	No skipping
126	H31D(+04-20)	GUU UCU GAA AUA ACA UAU ACC UGU	Skipping to 300 nM

Table 26

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 32

Antisense oligonucleotides directed at exon 32 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

- 5 H32D(+04-16) [SEQ ID NO:127] and H32A(+49+73) [SEQ ID NO:130] induced exon 32 skipping when delivered into cells at a concentration of 300 nM. Table 27 below also shows other antisense molecules tested at a concentration of 100 and 300 nM. These antisense molecules did not show an ability to induce exon skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
127	H32D(+04-16)	CAC CAG AAA UAC AUA CCA CA	Skipping to 300 nM
128	H32A(+151+170)	CAA UGA UUU AGC UGU GAC UG	No skipping
129	H32A(+10+32)	CGA AAC UUC AUG GAG ACA UCU UG	No skipping
130	H32A(+49+73)	CUU GUA GAC GCU GCU CAA AAU UGG C	Skipping to 300 nM

10 Table 27

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 33

Antisense oligonucleotides directed at exon 33 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

- 15 Figure 14 shows differing efficiencies of two antisense molecules directed at exon 33 acceptor splice site. H33A(+64+88) [SEQ ID NO:134] substantially induced exon 33 skipping when delivered into cells at a concentration of 10 nM. Table 28 below includes other antisense molecules tested at a concentration of 100, 200 and 300 nM. These antisense molecules showed a variable ability to induce exon skipping.

20

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
131	H33D(+09-11)	CAU GCA CAC ACC UUU GCU CC	No skipping
132	H33A(+53+76)	UCU GUA CAA UCU GAC GUC CAG UCU	Skipping to 200 nM
133	H33A(+30+56)	GUG UUU AUC ACC AUU UCC ACU UCA GAC	Skipping to 200 nM
134	H33A(+64+88)	GCG UCU GCU UUU UCU GUA CAA UCU G	Skipping to 10 nM

Table 28

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 34

Antisense oligonucleotides directed at exon 34 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Table 29 below includes antisense molecules tested at a concentration of 100 and 300 nM. These antisense molecules showed a variable ability to induce exon skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
135	H34A(+83+104)	UCC AUA UCU GUA GCU GGC AGC C	No skipping
136	H34A(+143+165)	CCA GGC AAC UUC AGA AUC CAA AU	No skipping
137	H34A(-20+10)	UUU CUG UUA CCU GAA AAG AAU UAU AAU GAA	Not tested
138	H34A(+46+70)	CAU UCA UUU CCU UUC GCA UCU UAC G	Skipping to 300 nM
139	H34A(+95+120)	UGA UCU CUU UGU CAA UUC CAU AUC UG	Skipping to 300 nM
140	H34D(+10-20)	UUC AGU GAU AUA GGU UUU ACC UUU CCC CAG	Not tested
141	H34A(+72+96)	CUG UAG CUG CCA GCC AUU CUG UCA AG	No skipping

Table 29

10

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 35

Antisense oligonucleotides directed at exon 35 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

5 Figure 15 shows differing efficiencies of antisense molecules directed at exon 35 acceptor splice site. H35A(+24+43) [SEQ ID NO:144] substantially induced exon 35 skipping when delivered into cells at a concentration of 20 nM. Table 30 below also includes other antisense molecules tested at a concentration of 100 and 300 nM. These antisense molecules showed no ability to induce exon skipping.

10

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
142	H35A(+141+161)	UCU UCU GCU CGG GAG GUG ACA	Skipping to 20 nM
143	H35A(+116+135)	CCA GUU ACU AUU CAG AAG AC	No skipping
144	H35A(+24+43)	UCU UCA GGU GCA CCU UCU GU	No skipping

Table 30

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 36

Antisense oligonucleotides directed at exon 36 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

15 described above.

Antisense molecule H36A(+26+50) [SEQ ID NO:145] induced exon 36 skipping when delivered into cells at a concentration of 300 nM, as shown in Figure 16.

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 37

Antisense oligonucleotides directed at exon 37 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as

20

described above. Figure 17 shows differing efficiencies of two antisense molecules directed at exon 37 acceptor splice site. H37A(+82+105) [SEQ ID NO:148] and H37A(+134+157)

[SEQ ID NO:149] substantially induced exon 37 skipping when delivered into cells at a concentration of 10 nM. Table 31 below shows the antisense molecules tested.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
147	H37A(+26+50)	CGU GUA GAG UCC ACC UUU GGG CGU A	No skipping
148	H37A(+82+105)	UAC UAA UUU CCU GCA GUG GUC ACC	Skipping to 10 nM
149	H37A(+134+157)	UUC UGU GUG AAA UGG CUG CAA AUC	Skipping to 10 nM

Table 31

5 ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 38

Antisense oligonucleotides directed at exon 38 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 18 illustrates antisense molecule H38A(+88+112) [SEQ ID NO:152], directed at exon 38 acceptor splice site. H38A(+88+112) substantially induced exon 38 skipping when delivered into cells at a concentration of 10 nM. Table 32 below shows the antisense molecules tested and their ability to induce exon skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
150	H38A(-01+19)	CCU UCA AAG GAA UGG AGG CC	No skipping
151	H38A(+59+83)	UGC UGA AUU UCA GCC UCC AGU GGU U	Skipping to 10 nM
152	H38A(+88+112)	UGA AGU CUU CCU CUU UCA GAU UCA C	Skipping to 10 nM

Table 32

15 ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 39

Antisense oligonucleotides directed at exon 39 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H39A(+62+85) [SEQ ID NO:153] induced exon 39 skipping when delivered into cells at a concentration of 100 nM. Table 33 below shows the antisense molecules tested and their ability to induce exon skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
153	H39A(+62+85)	CUG GCU UUC UCU CAU CUG UGA UUC	Skipping to 100 nM
154	H39A(+39+58)	GUU GUA AGU UGU CUC CUC UU	No skipping
155	H39A(+102+121)	UUG UCU GUA ACA GCU GCU GU	No skipping
156	H39D(+10-10)	GCU CUA AUA CCU UGA GAG CA	Skipping to 300 nM

5 Table 33

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 40

Antisense oligonucleotides directed at exon 40 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

- 10 Figure 19 illustrates antisense molecule H40A(-05+17) [SEQ ID NO:157] directed at exon 40 acceptor splice site. H40A(-05+17) and H40A(+129+153) [SEQ ID NO:158] both substantially induced exon 40 skipping when delivered into cells at a concentration of 5 nM.

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 42

- 15 Antisense oligonucleotides directed at exon 42 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

- 20 Figure 20 illustrates antisense molecule H42A(-04+23) [SEQ ID NO:159], directed at exon 42 acceptor splice site. H42A(-4+23) and H42D(+19-02) [SEQ ID NO:161] both induced exon 42 skipping when delivered into cells at a concentration of 5 nM. Table 34 below shows the antisense molecules tested and their ability to induce exon 42 skipping.

SEQ ID	Antisense afigonucleotide name	Sequence	Ability to induce skipping
159	H42A(-4+23)	AUC GUU UCU UCA CGG ACA GUG UGG UGC	Skipping to 5 nM
160	H42A(+86+109)	GGG CUU GUG AGA CAU GAG UGA UUU	Skipping to 100 nM
161	H42D(+19-02)	A CCU UCA GAG GAC UCC UCU UGC	Skipping to 5 nM

Table 34

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 43

Antisense oligonucleotides directed at exon 43 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H43A(+101+120) [SEQ ID NO:163] induced exon 43 skipping when delivered into cells at a concentration of 25 nM. Table 35 below includes the antisense molecules tested and their ability to induce exon 43 skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
162	H43D(+10-15)	UAU GUG UUA CCU ACC CUU GUC GGU C	Skipping to 100 nM
163	H43A(+101+120)	GGA GAG AGC UUC CUG UAG CU	Skipping to 25 nM
164	H43A(+78+100)	UCA CCC UUU CCA CAG GCG UUG CA	Skipping to 200 n M

Table 35

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 44

Antisense oligonucleotides directed at exon 44 were prepared using similar methods as described above. Testing for the ability of these antisense molecules to induce exon 44 skipping is still in progress. The antisense molecules under review are shown as

SEQ ID Nos: 165 to 167 in Table 1A.

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 45

Antisense oligonucleotides directed at exon 45 were prepared using similar methods as described above. Testing for the ability of these antisense molecules to induce exon 45 skipping is still in progress. The antisense molecules under review are shown as

5 SEQ ID Nos: 207 to 211 in Table 1A.

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 46

Antisense oligonucleotides directed at exon 46 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

10 Figure 21 illustrates the efficiency of one antisense molecule directed at exon 46 acceptor splice site. Antisense oligonucleotide H46A(+86+115) [SEQ ID NO:203] showed very strong ability to induce exon 46 skipping. Table 36 below includes antisense molecules tested. These antisense molecules showed varying ability to induce exon 46 skipping.

15

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
168	H46D(+16-04)	UUA CCU UGA CUU GCU CAA GC	No skipping
169	H46A(+90+109)	UCC AGG UUC AAG UGG GAU AC	No skipping
203	H46A(+86+115)	CUC UUU UCC AGG UUC AAG UGG GAU ACU AGC	Good skipping to 100 nM
204	H46A(+107+137)	CAA GCU UUU CUU UUA GUU GCU GCU CUU UUC C	Good skipping to 100 nM
205	H46A(-10+20)	UAU UCU UUU GUU CUU CUA GCC UGG AGA AAG	Weak skipping
206	H46A(+50+77)	CUG CUU CCU CCA ACC AUA AAA CAA AUU C	Weak skipping

Table 36

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 47

Antisense oligonucleotides directed at exon 47 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

- 5 H47A(+76+100) [SEQ ID NO:170] and H47A(-09+12) [SEQ ID NO:172] both induced exon 47 skipping when delivered into cells at a concentration of 200 nM. H47D(+25-02) [SEQ ID NO: 171] is yet to be prepared and tested.

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 50

- 10 Antisense oligonucleotides directed at exon 50 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

- Antisense oligonucleotide molecule H50A(+02+30) [SEQ ID NO: 173] was a strong inducer of exon skipping. Further, H50A(+07+33) [SEQ ID NO:174] and H50D(+07-18) [SEQ ID NO:175] both induced exon 50 skipping when delivered into cells
15 at a concentration of 100 nM.

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 51

Antisense oligonucleotides directed at exon 51 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

- 20 Figure 22 illustrates differing efficiencies of two antisense molecules directed at exon 51 acceptor splice site. Antisense oligonucleotide H51A(+66+90) [SEQ ID NO:180] showed the stronger ability to induce exon 51 skipping. Table 37 below includes antisense molecules tested at a concentration range of 25, 50, 100, 300 and 600 nM. These antisense molecules showed varying ability to induce exon 51 skipping. The
25 strongest inducers of exon skipping were antisense oligonucleotide H51A(+61+90) [SEQ ID NO: 179] and H51A(+66+95) [SEQ ID NO: 181].

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
176	H51A(-01+25)	ACC AGA GUA ACA GUC UGA GUA GGA GC	Faint skipping
177	H51D(+16-07)	CUC AUA CCU UCU GCU UGA UGA UC	Skipping at 300 nM
178	H51A(+111+134)	UUC UGU CCA AGC CCG GUU GAA AUC	Needs re- testing
179	H51A(+61+90)	ACA UCA AGG AAG AUG GCA UUU CUA GUU UGG	Very strong skipping
180	H51A(+66+90)	ACA UCA AGG AAG AUG GCA UUU CUA G	skipping
181	H51A(+66+95)	CUC CAA CAU CAA GGA AGA UGG CAU UUC UAG	Very strong skipping
182	H51D(+08-17)	AUC AUU UUU UCU CAU ACC UUC UGC U	No skipping
183	H51A/D(+08-17) & (-15+?)	AUC AUU UUU UCU CAU ACC UUC UGC UAG GAG CUA AAA	No skipping
184	H51A(+175+195)	CAC CCA CCA UCA GCC UCU GUG	No skipping
185	H51A(+199+220)	AUC AUC UCG UUG AUA UCC UCA A	No skipping

Table 37

ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 52

Antisense oligonucleotides directed at exon 52 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 22 also shows differing efficiencies of four antisense molecules directed at exon 52 acceptor splice site. The most effective antisense oligonucleotide for inducing exon 52 skipping was H52A(+17+37) [SEQ ID NO:188).

Table 38 below shows antisense molecules tested at a concentration range of 50, 100, 300 and 600 nM. These antisense molecules showed varying ability to induce exon 50 skipping. Antisense molecules H52A(+12+41) [SEQ ID NO:187] and

H52A(+17+37) [SEQ ID NO:188] showed the strongest exon 50 skipping at a concentration of 50 nM.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
186	H52A(-07+14)	UCC UGC AUU GUU GCC UGU AAG	No skipping
187	H52A(+12+41)	UCC AAC UGG GGA CGC CUC UGU UCC AAA UCC	Very strong skipping
188	H52A(+17+37)	ACU GGG GAC GCC UCU GUU CCA	Skipping to 50 nM
189	H52A(+93+112)	CCG UAA UGA UUG UUC UAG CC	No skipping
190	H52D(+05-15)	UGU UAA AAA ACU UAC UUC GA	No skipping

Table 38

5 ANTISENSE OLIGONUCLEOTIDES DIRECTED AT EXON 53

Antisense oligonucleotides directed at exon 53 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 22 also shows antisense molecule H53A(+39+69) [SEQ ID NO:193] directed at exon 53 acceptor splice site. This antisense oligonucleotide was able to induce exon 53 skipping at 5, 100, 300 and 600 nM. A "cocktail" of three exon 53 antisense oligonucleotides: H53A(+23+47) [SEQ ID NO:195], H53A(+150+176) [SEQ ID NO:196] and H53D(+14-07) [SEQ ID NO:194], was also tested, as shown in Figure 20 and exhibited an ability to induce exon skipping.

Table 39 below includes other antisense molecules tested at a concentration range of 50, 100, 300 and 600 nM. These antisense molecules showed varying ability to induce exon 53 skipping. Antisense molecule H53A(+39+69) [SEQ ID NO:193] induced the strongest exon 53 skipping.

SEQ ID	Antisense oligonucleotide name	Sequence	Ability to induce skipping
191	H53A(+45+69)	CAU UCA ACU GUU GCC UCC GGU UCU G	Faint skipping at 50 nM
192	H53A(+39+62)	CUG UUG CCU CCG GUU CUG AAG GUG	Faint skipping at 50 nM
193	H53A(+39+69)	CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G	Strong skipping to 50 nM
194	H53D(+14-07)	UAC UAA CCU UGG UUU CUG UGA	Very faint skipping to 50 nM
195	H53A(+23+47)	CUG AAG GUG UUC UUG UAC UUC AUC C	Very faint skipping to 50 nM
196	H53A(+150+176)	UGU AUA GGG ACC CUC CUU CCA UGA CUC	Very faint skipping to 50 nM
197	H53D(+20-05)	CUA ACC UUG GUU UCU GUG AUU UUC U	Not made yet
198	H53D(+09-18)	GGU AUC UUU GAU ACU AAC CUU GGU UUC	Faint at 600 nM
199	H53A(-12+10)	AUU CUU UCA ACU AGA AUA AAA G	No skipping
200	H53A(-07+18)	GAU UCU GAA UUG UUU CAA CUA GAA U	No skipping
201	H53A(+07+26)	AUC CCA CUG AUU CUG AAU UC	No skipping
202	H53A(+124+145)	UUG GCU CUG GCC UGU CCU AAG A	No skipping

Table 39

What is claimed is:

1. A method for treating a patient with Duchenne muscular dystrophy (DMD) in need thereof who has a mutation of the DMD gene that is amenable to exon 53 skipping,
5 comprising administering to the patient an antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC
10 UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.
- 15 2. The method of claim 2, wherein the antisense oligonucleotide is administered intravenously.

20

25

30

ABSTRACT

An antisense molecule capable of binding to a selected target site to induce exon skipping in the dystrophin gene, as set forth in SEQ ID NO: 1 to 214.

5

10

FIGURE 1

1/22

Donor

ESE

Acceptor

bp

udaugcacugagugacccuuucucgcagGCGCUAGCUGGAGCA////CCGUGCAGACUGACGgucucau

SEQ ID NO:214

SEQ ID NO:213

2/22

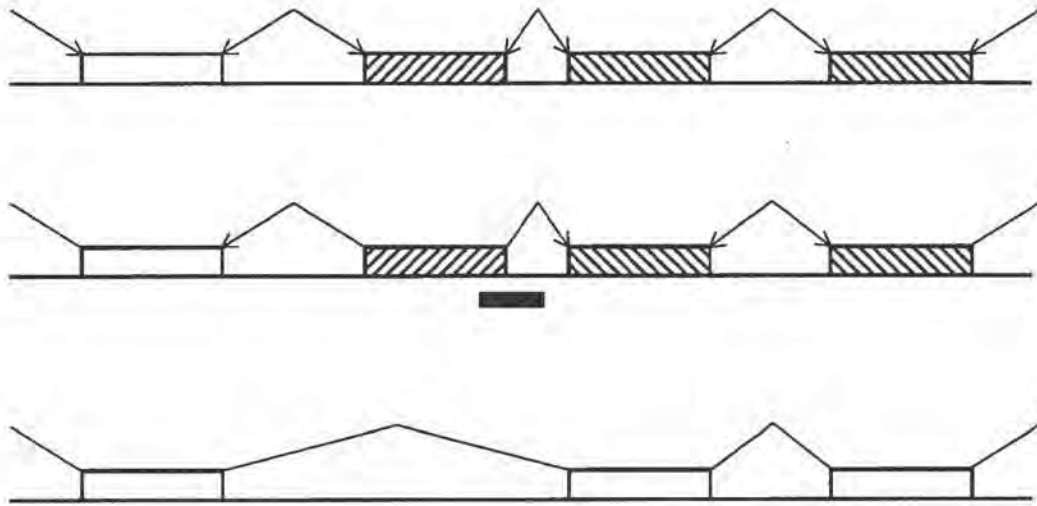


FIGURE 2

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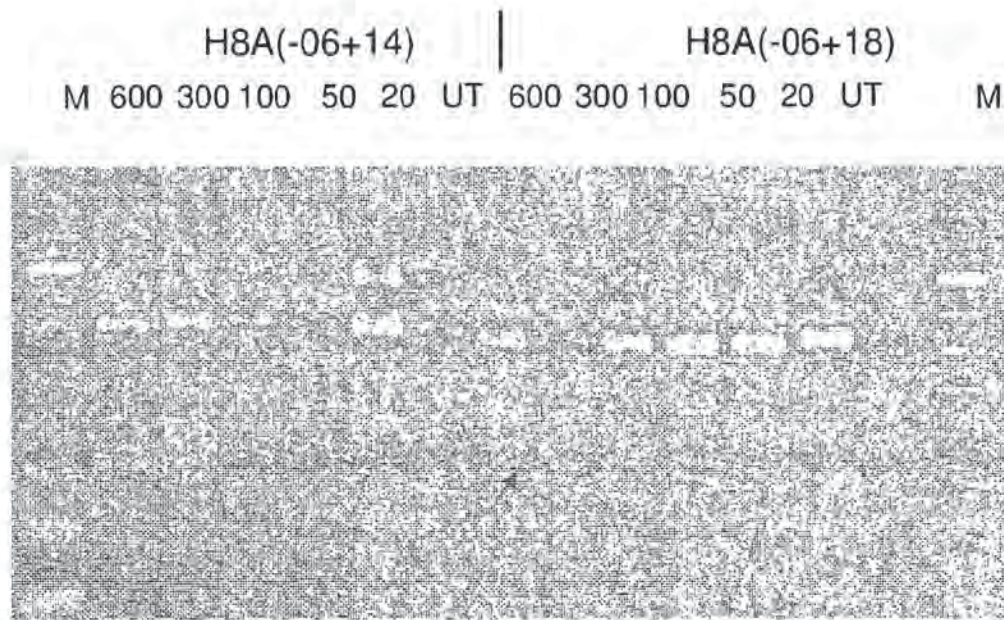


FIGURE 3

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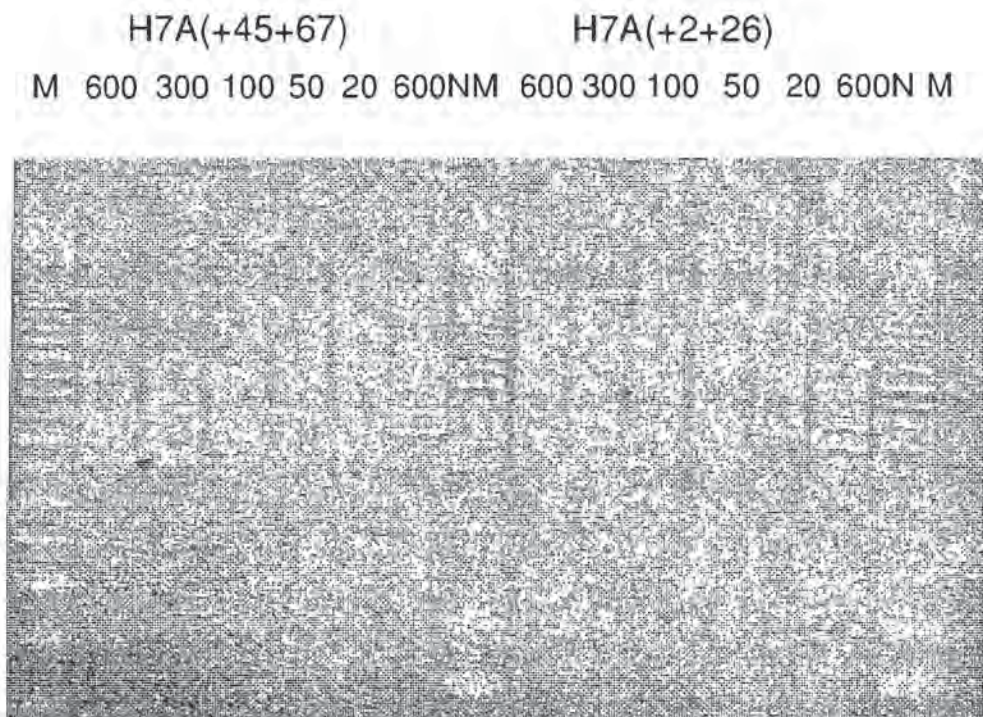


FIGURE 4

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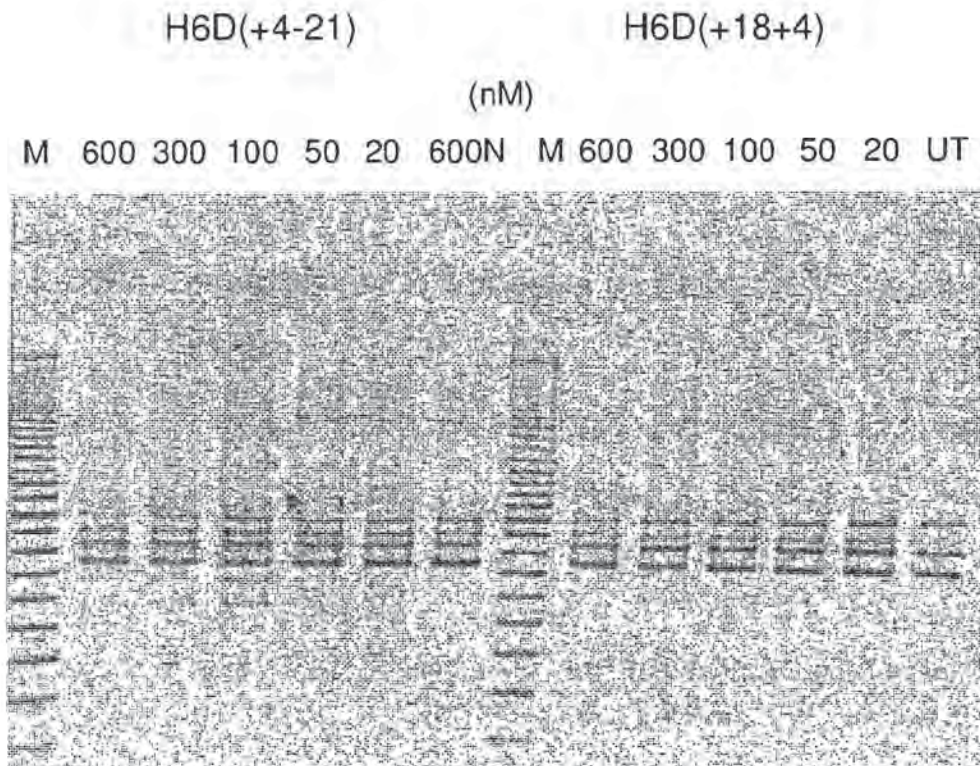


FIGURE 5

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6A(+69+91)

M 600 300 200 100 50 20 UT

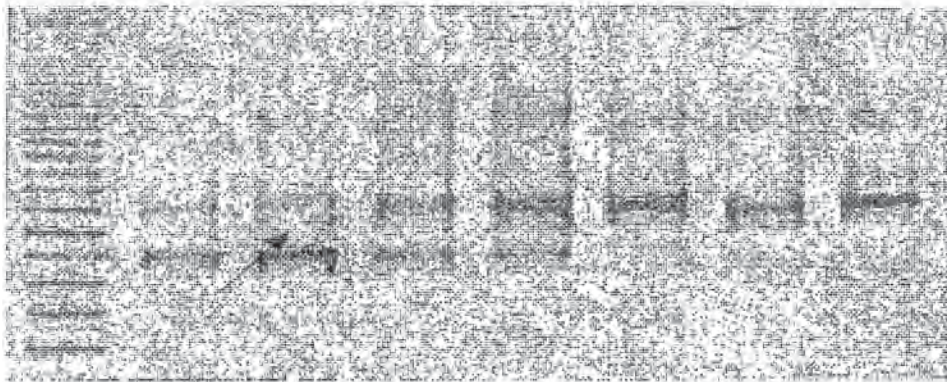


FIGURE 6

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H4A(+13+32)

M 600 300 100 50 20 UT Neg M

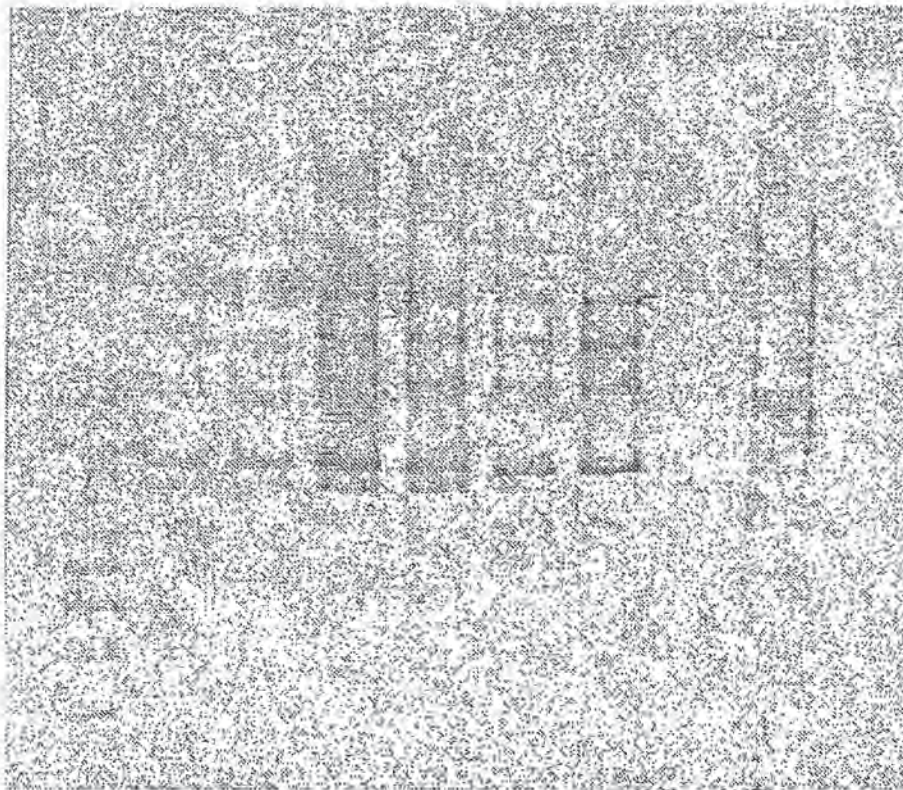


FIGURE 7

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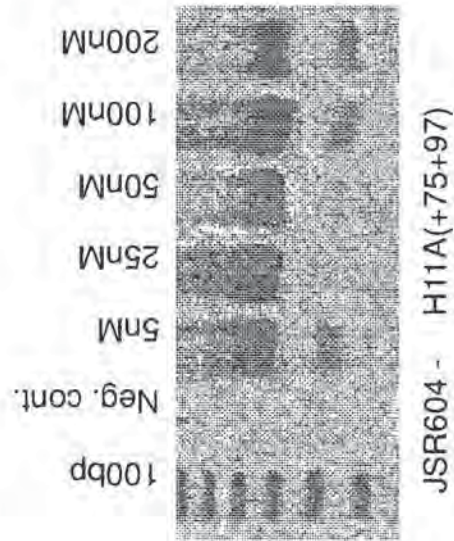


FIGURE 8B

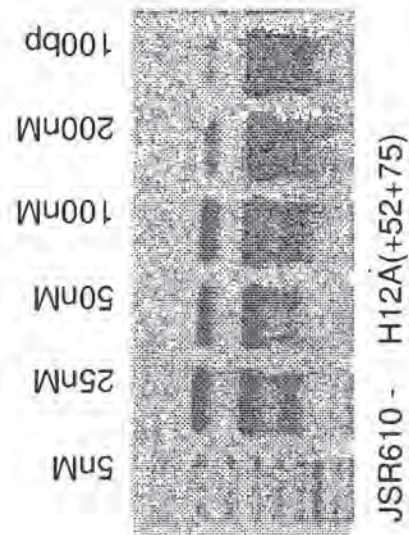


FIGURE 8A

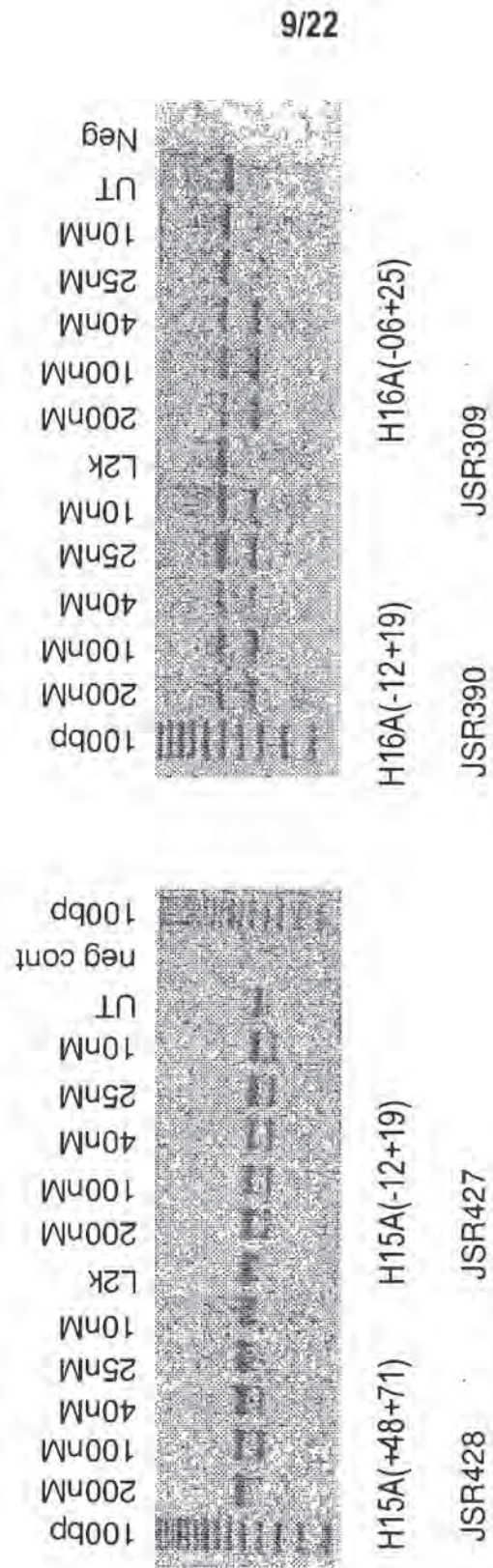


FIGURE 9A

FIGURE 9B

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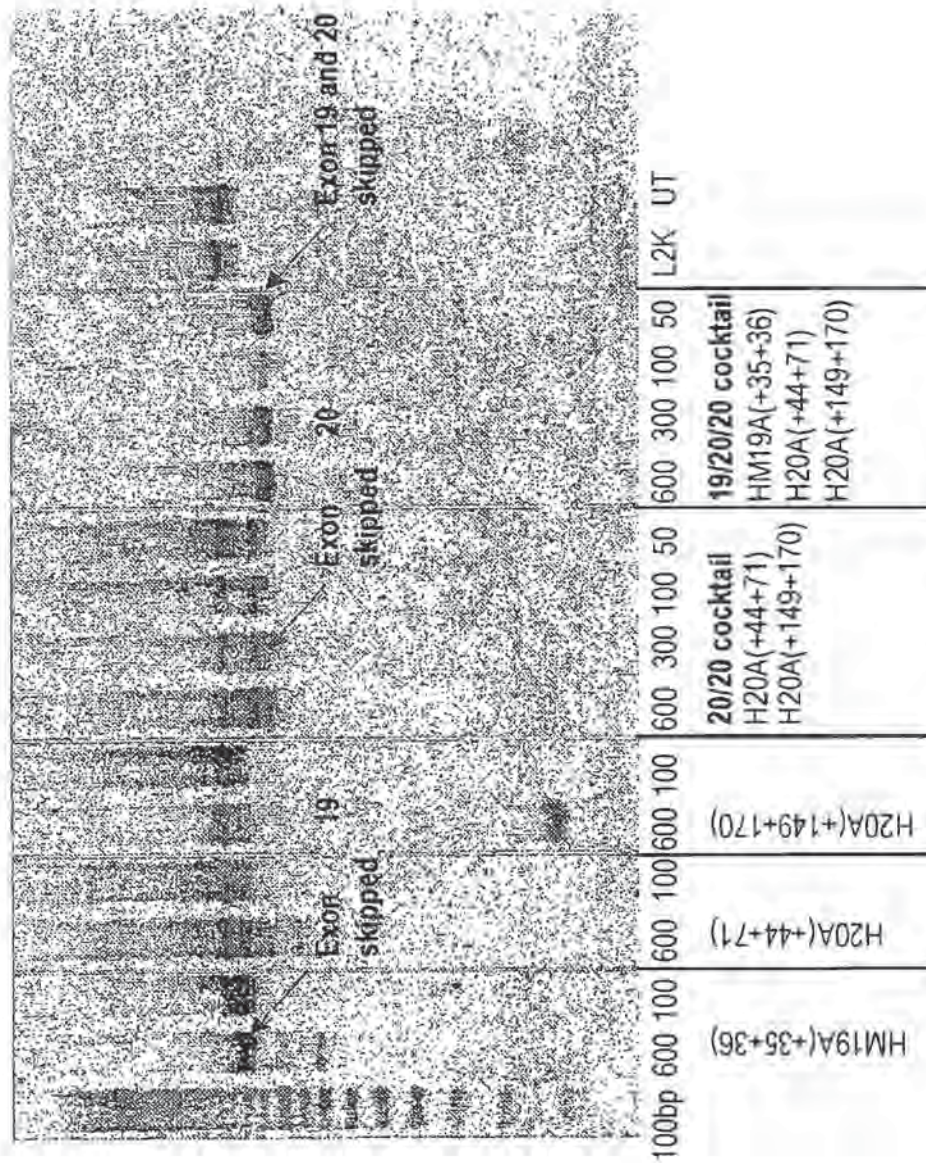
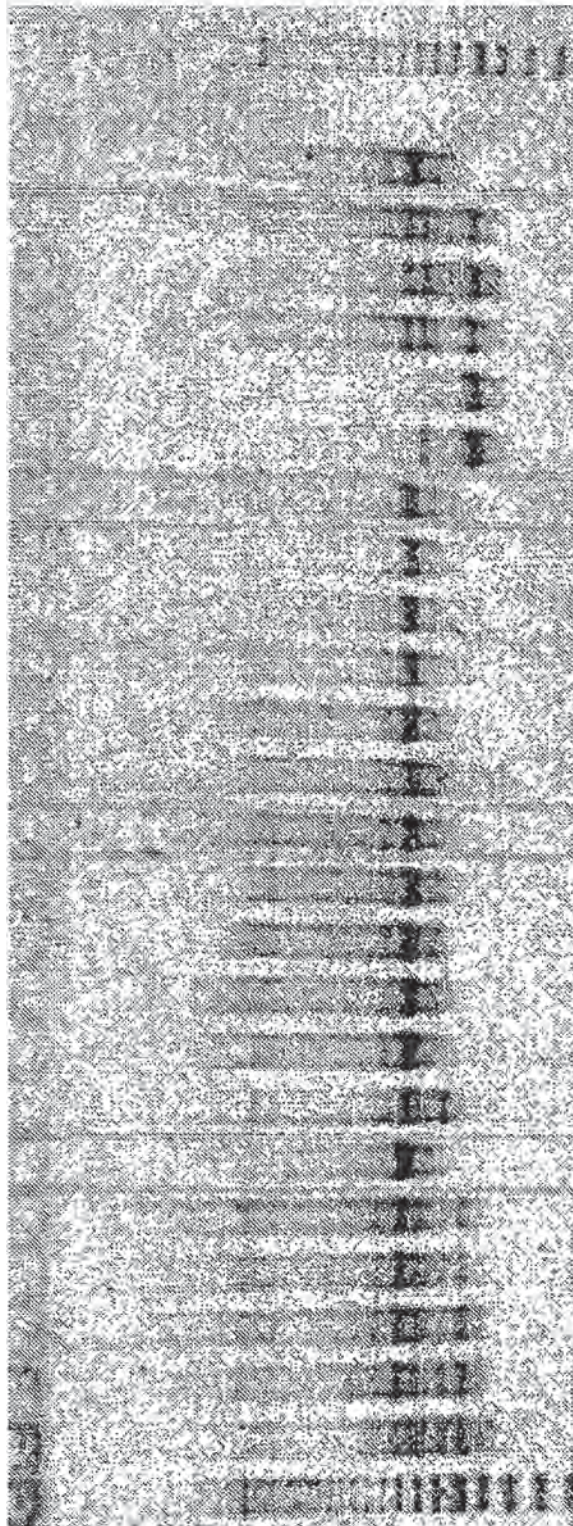


FIGURE 10

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19/20/20 cocktail
HM19A(+35+36)
H20A(+44+71)
H20A(+149+170)

Weasel19/20
H19A(+35+53)-
aa-
H20A(+149+168)

Weasel19/20
H19A(+35+53)-
aa-
H20A(+44+63)

Weasel19/20/20
H19A(+35+53)-aa-
H20A(+44+63)-aa-
H20A(+149+168)

FIGURE 11

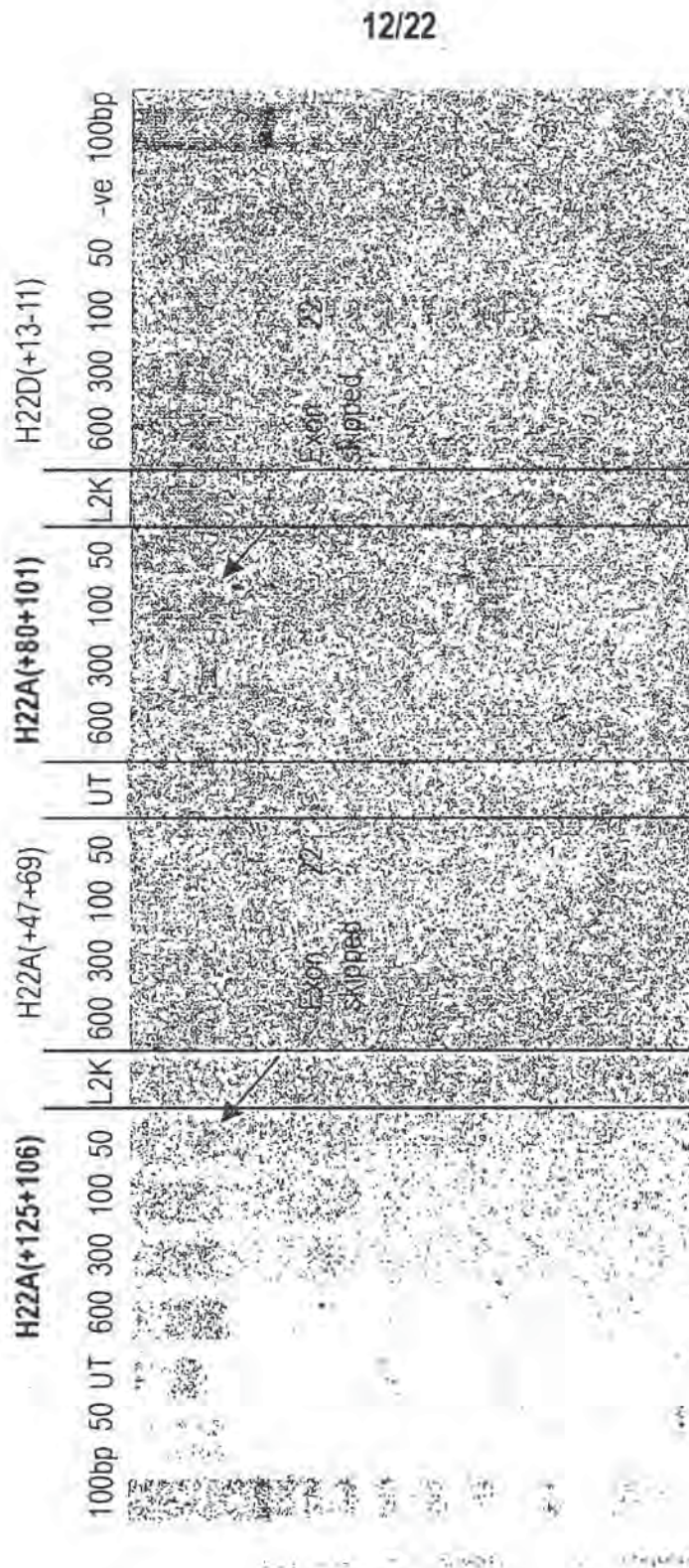


FIGURE 12



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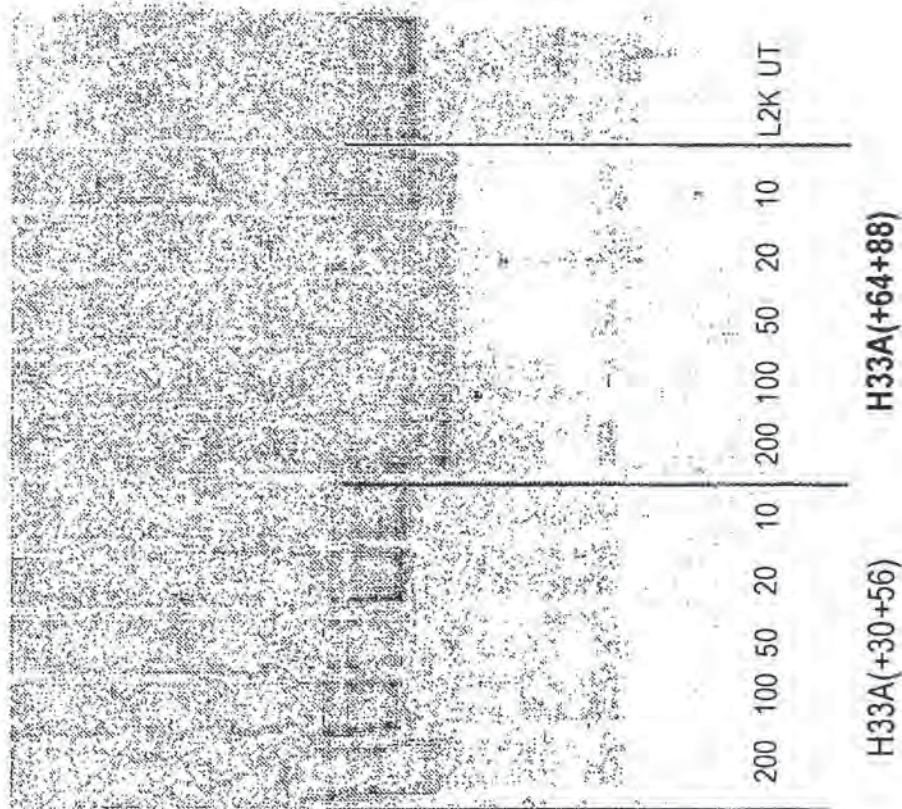


FIGURE 14

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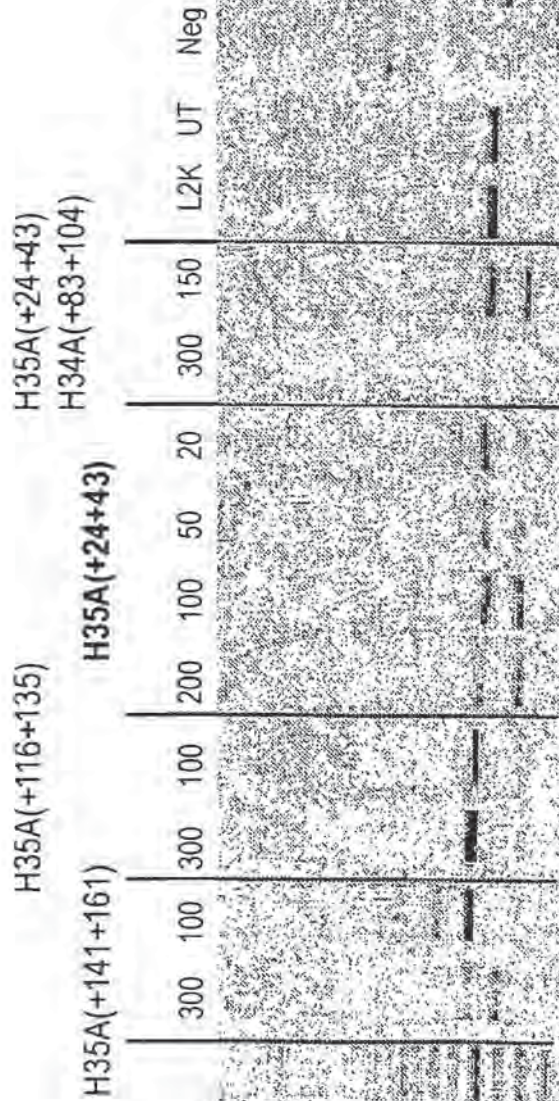


FIGURE 15

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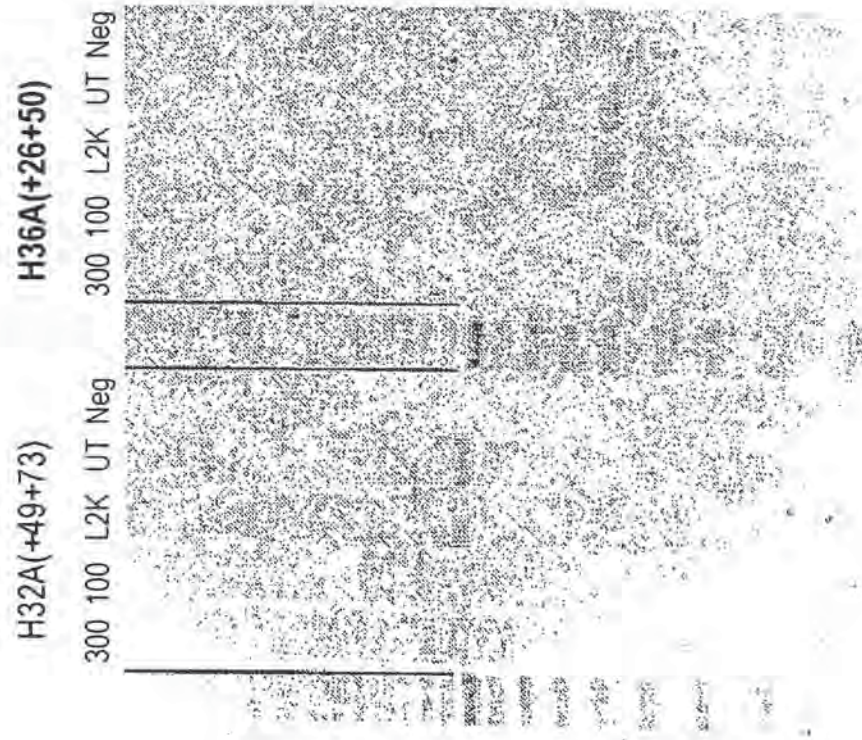


FIGURE 16

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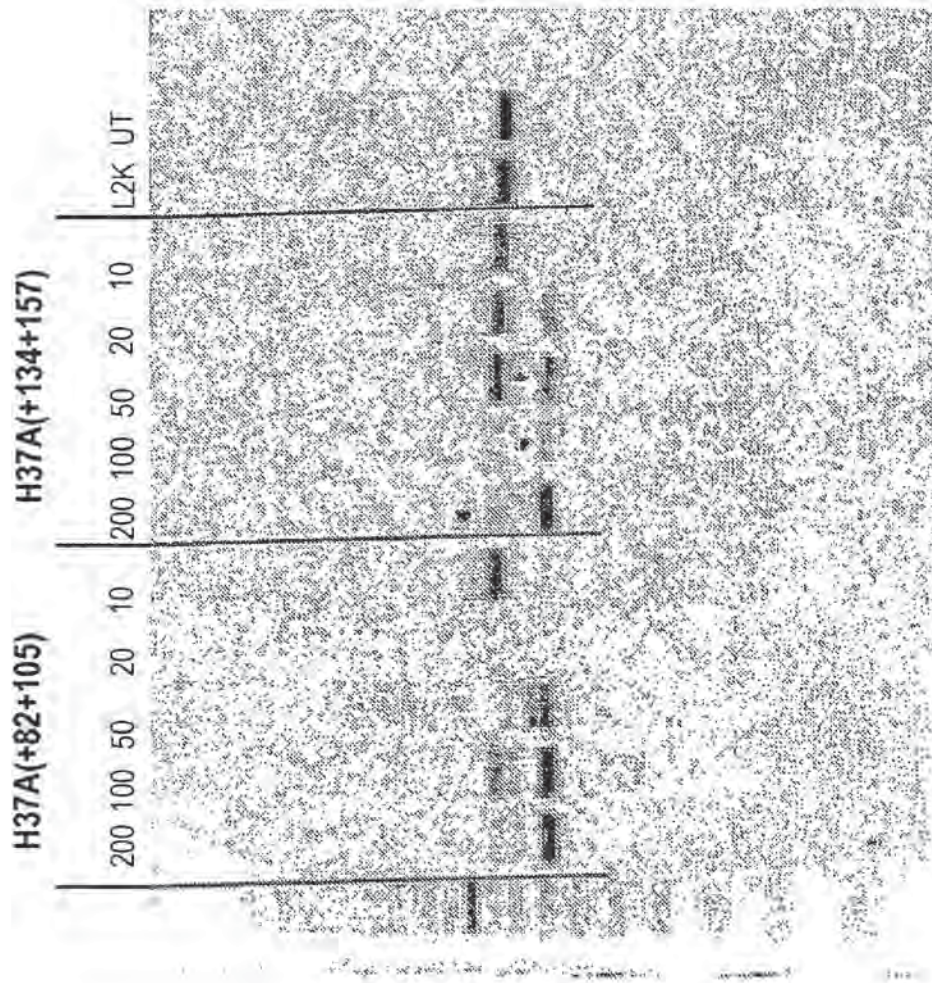
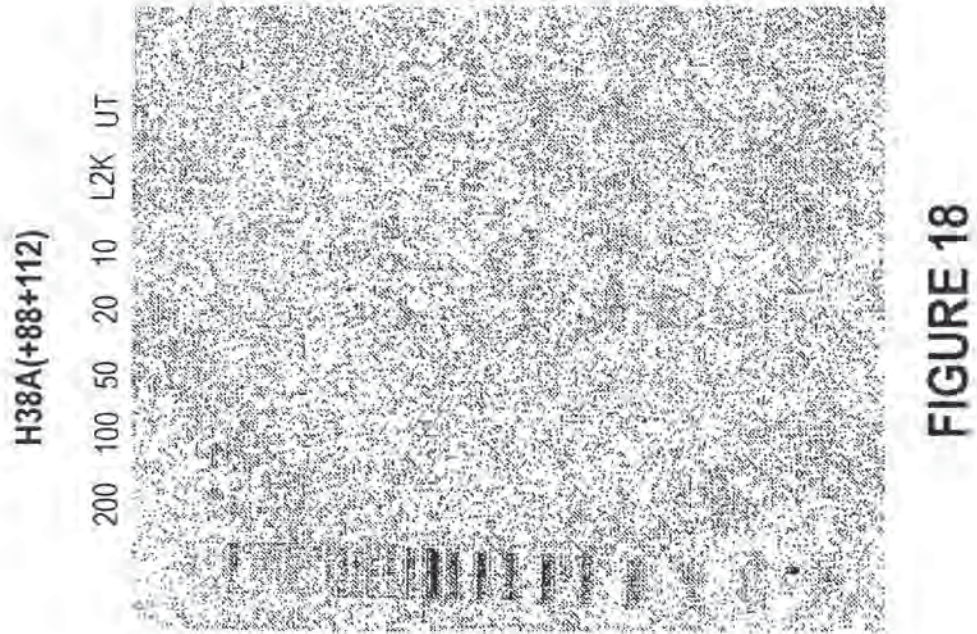


FIGURE 17

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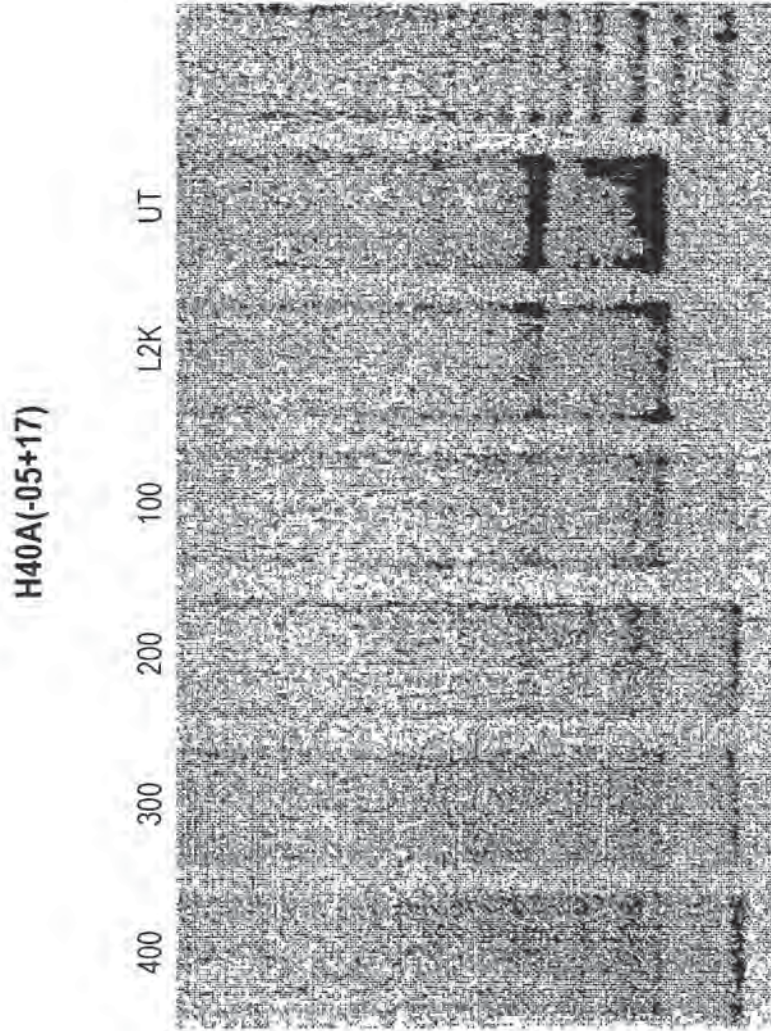


FIGURE 19

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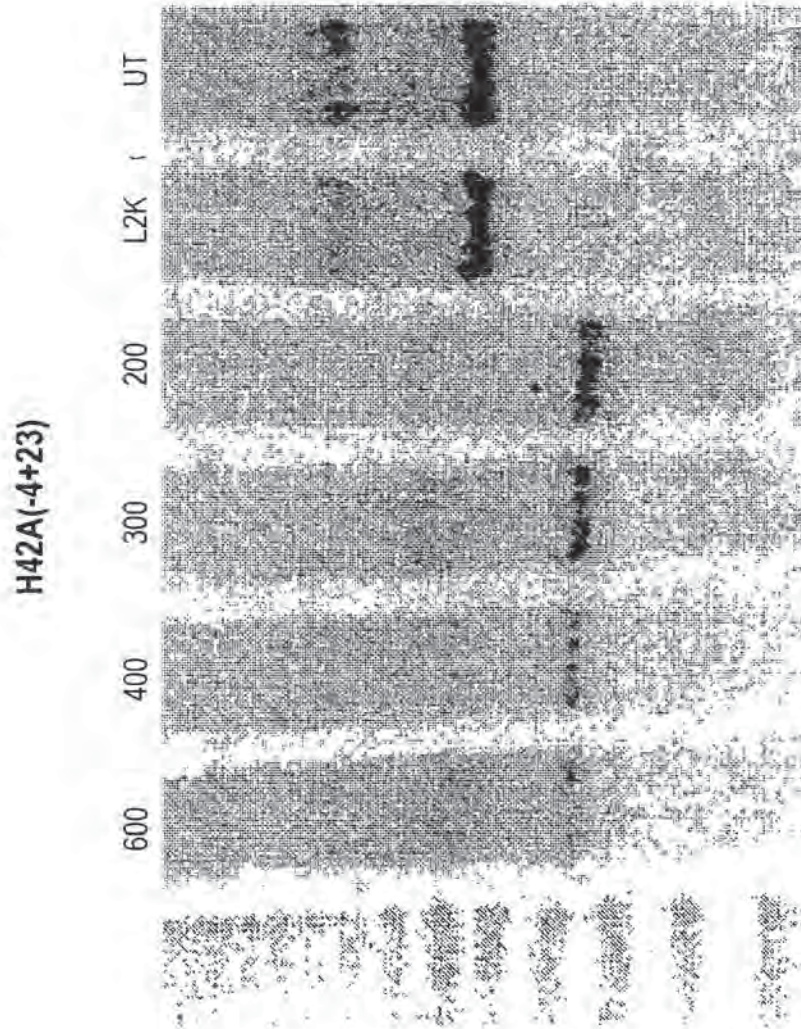


FIGURE 20

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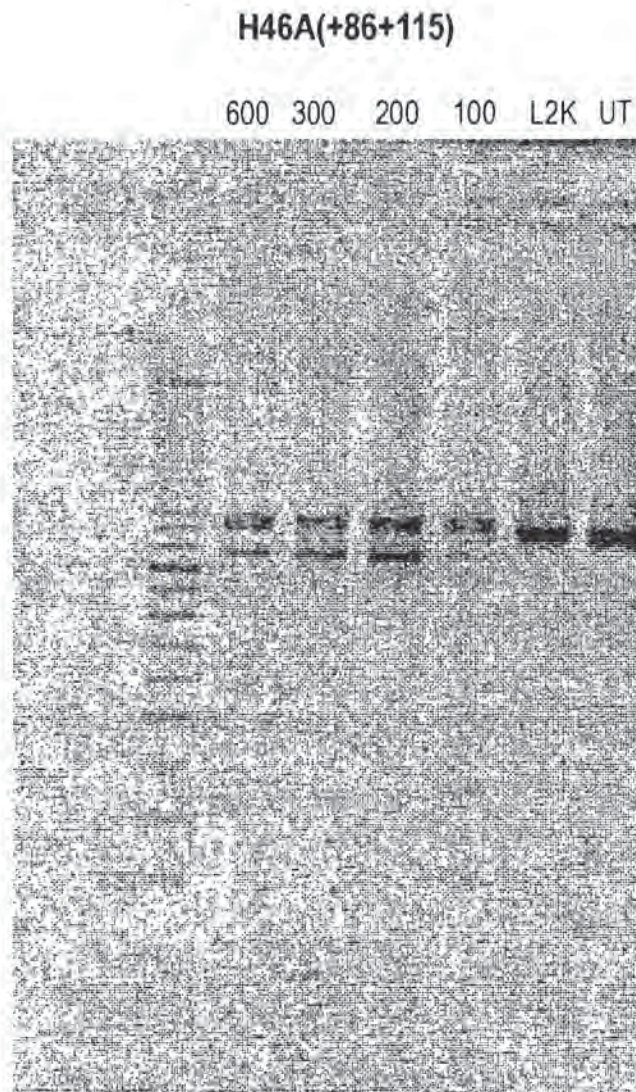


FIGURE 21

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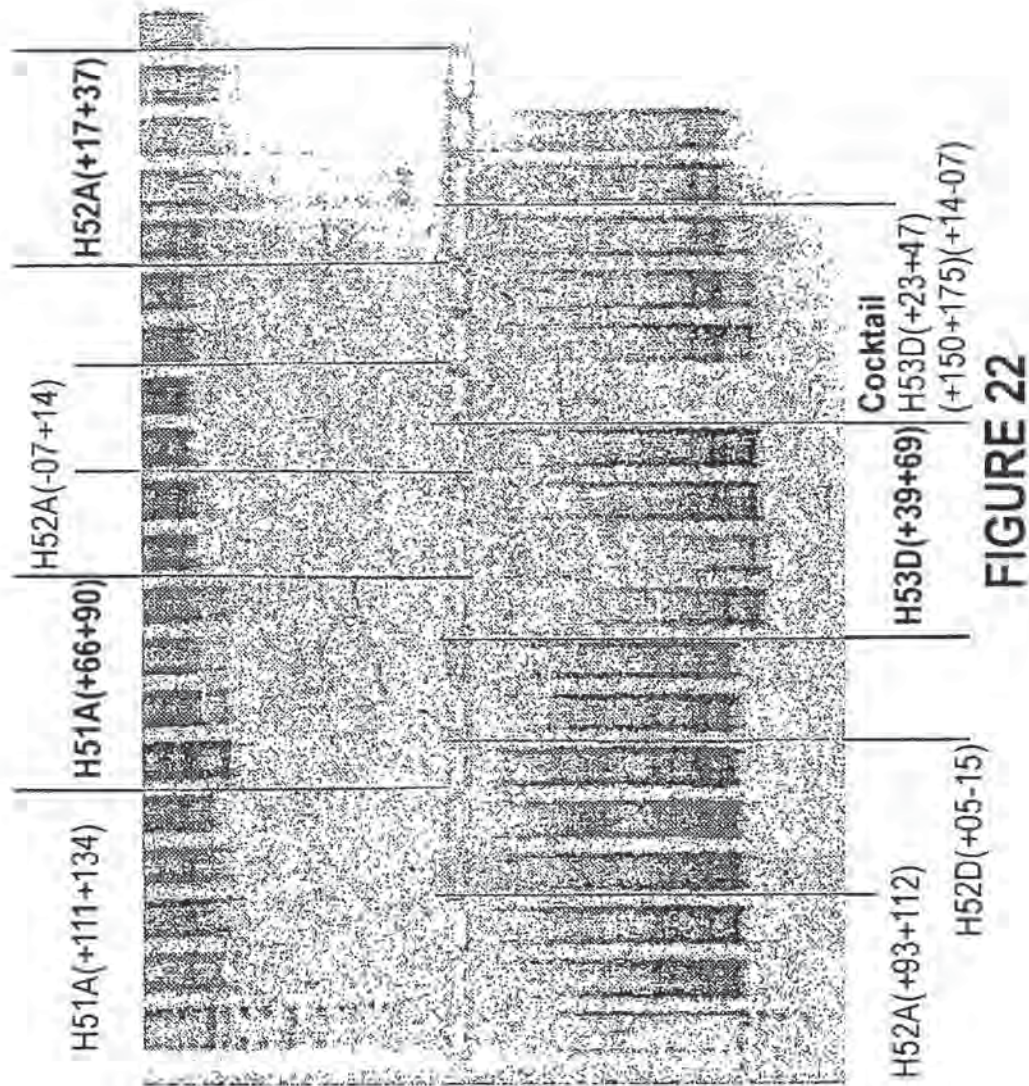


FIGURE 22



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/112,453	08/24/2018	Stephen Donald WILTON	4140.01500B1	3144
153767	7590	12/28/2018	EXAMINER	
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.			CHONG, KIMBERLY	
1100 NEW YORK AVENUE, N.W.			ART UNIT	
WASHINGTON, DC 20005			PAPER NUMBER	
			1635	
			NOTIFICATION DATE	
			DELIVERY MODE	
			12/28/2018	
			ELECTRONIC	

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

e-office@sternekessler.com
jcovert@sternekessler.com

Office Action Summary**Application No.**

16/112,453

Applicant(s)

WILTON et al.

Examiner

KIMBERLY CHONG

Art Unit

1635

AIA Status

No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
 - If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
 - Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 11/29/2013.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

- 5) ☒ Claim(s) 1-2 is/are pending in the application.
 5a) Of the above claim(s) ____ is/are withdrawn from consideration.
- 6) ☐ Claim(s) ____ is/are allowed.
- 7) ☒ Claim(s) 1-2 is/are rejected.
- 8) ☐ Claim(s) ____ is/are objected to.
- 9) ☐ Claim(s) ____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

- 10) ☐ The specification is objected to by the Examiner.
- 11) ☒ The drawing(s) filed on 08/24/2018 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

- 12) ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

- a) ☒ All b) ☐ Some** c) ☐ None of the:
- ☐ Certified copies of the priority documents have been received.
 - ☒ Certified copies of the priority documents have been received in Application No. 11570691.
 - ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☐ Notice of References Cited (PTO-892)
- 2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)
 Paper No(s)/Mail Date ____.
- 3) ☐ Interview Summary (PTO-413)
 Paper No(s)/Mail Date ____.
- 4) ☐ Other: ____.

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Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Status of the Application

Claims 1 and 2 are pending and are currently under examination.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):

(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 1-2 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

The claims recite that the target region is "within" the two annealing sites H53A(+23+47) and H53A(+39+69). The target region within the annealing sites H53A(+23+47) and H53A(+39+69) is 9 base long (i.e., 47-39), whereas the claimed antisense molecule is 20 to 31 bases long and is 100 % complementary to consecutive nucleotides of the target region, and comprises at least 12 consecutive bases of the sequence of SEQ ID NO: 195. Because the target region within the two listed annealing sites is 9 base long, it is not clear how a 20 to 31 base oligonucleotide would be 100%

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complementary to consecutive nucleotides of the target region. Thus, the meets and bounds of the claimed antisense oligonucleotide is not clear.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(a):

(a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), first paragraph:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1 and 2 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for methods of inducing exon skipping in muscle cells using an antisense molecule H53A(+39+69) [SEQ ID NO:193] directed at exon 53 acceptor splice site, is not enabling for a method for treating a patient with Duchenne muscular dystrophy (DMD) in need thereof who has a mutation of the DMD gene that is amenable to exon 53 skipping, comprising administering to the patient an antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69).

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The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

The following factors have been considered in the analysis of enablement: (1) the breadth of the claims, (2) the nature of the invention, (3) the state of the prior art, (4) the level of one of ordinary skill, (5) the level of predictability in the art, (6) the amount of direction provided by the inventor, (7) the existence of working examples, (8) the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

The instant claims are drawn to a method of treating a patient with Duchenne muscular dystrophy (DMD) in need thereof who has a mutation of the DMD gene that is amenable to exon 53 skipping, comprising administering to the patient an antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a ~~non-bulky antisense oligonucleotide, and wherein the antisense oligonucleotide~~

<220>

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oligonucleotide up to 31 nucleotides in length that only binds a target region within the annealing sites H53A(+23+47) and H53A(+39+69, which is 9 base long (i.e., 47-39).

Whether the specification would have been enabling as of the filing date involves consideration of the nature of the invention, the state of the prior art, and the level of skill in the art. The state of the prior art is what one skilled in the art would have known, at the time the application was filed, about the subject matter to which the claimed invention pertains. The relative skill of those in the art refers to the skill of those in the art in relation to the subject matter to which the claimed invention pertains at the time the application was filed. See MPEP § 2164.05(b). The state of the prior art provides evidence for the degree of predictability in the art and is related to the amount of direction or guidance needed in the specification as filed to meet the enablement requirement. The state of the prior art is also related to the need for working examples in the specification.

A thorough review of the patent and non-patent literature indicates that the state of the art demonstrating exon skipping for treatment of DMD using an antisense oligonucleotide 9 nucleotides in length targeting a specific target region was nascent at the time of filing of the instant application.

Thus while the prior does indicate that exon skipping using antisense oligonucleotides targeted to exons of the DMD gene, the prior art does not demonstrate that exon skipping using an antisense oligonucleotide up to 31 nucleotides in length wherein the oligonucleotide only binds within a 9 nucleotide target region. Because the state of the prior art does not provide evidence of the degree of predictability for the

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claimed method, one of ordinary skill in the art would look for guidance or direction in the instant specification.

The amount of guidance or direction needed to enable the invention is inversely related to the amount of knowledge in the state of the art as well as the predictability in the art. In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). The “amount of guidance or direction” refers to that information in the application, as originally filed, that teaches exactly how to make or use the invention. The more that is known in the prior art about the nature of the invention, how to make, and how to use the invention, and the more predictable the art is, the less information needs to be explicitly stated in the specification. In contrast, if little is known in the prior art about the nature of the invention and the art is unpredictable, the specification would need more detail as to how to make and use the invention in order to be enabling. >See, e.g., Chiron Corp. v. Genentech Inc., 363 F.3d 1247, 1254, 70 USPQ2d 1321, 1326 (Fed. Cir. 2004).

While the level of one of ordinary skill practicing said invention would be high, the level of predictability is considered variable as evident in the prior art discussed above and is not considered to provide sufficient enablement to practice the claimed invention.

The working embodiment in the instant application illustrates methods of inducing exon skipping in muscle cells using an antisense molecule H53A(+39+69) [SEQ ID NO:193] directed at exon 53 acceptor splice site. The working embodiment in the instant application does not include experiments demonstrating exon skipping using an antisense oligonucleotide up to 31 nucleotides in length wherein the oligonucleotide only binds within a 9 nucleotide target region. While the MPEP 2164.02 states the specification need not contain an example if the invention is otherwise disclosed in such

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manner that one skilled in the art will be able to practice it without an undue amount of experimentation. In *re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970), the lack of a working example, however, is a factor to be considered, especially in a case involving an unpredictable and undeveloped art.

Without further guidance, one of skill in the art would have to practice a substantial amount of trial and error experimentation, an amount considered undue and not routine, to practice the instantly claimed invention.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

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The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to <http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp>.

Claims 1 and 2 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-2 of U.S. Patent No. 9,994,851. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to antisense oligonucleotides having at least 12 consecutive bases of SEQ ID No. 195.

Claims 1 and 2 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-25 of U.S. Patent No. 8,232,384. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to antisense oligonucleotides having at least 12 consecutive bases of SEQ ID No. 195.

Claims 1 and 2 are provisionally rejected under the judicially created doctrine of double patenting over claims 44-64 of copending Application No. 15/645,842. This is a

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provisional double patenting rejection since the conflicting claims have not yet been patented. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the patent are drawn to antisense oligonucleotides having consecutive bases of SEQ ID No. 195.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **KIMBERLY CHONG** at (571)272-3111. The examiner can normally be reached Monday thru Friday between M-F 8:00am-4:30pm.

If attempts to reach the examiner by telephone are unsuccessful please contact the SPE for 1635 Ram Shukla at 571-272-0735. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system

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For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Primary Examiner
Art Unit 1635

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors: WILTON *et al.*

Confirmation No.: 3144

Applicant: The University of Western
Australia

Art Unit: 1635

Application No.: 16/112,453

Examiner: Chong, Kimberly

Filing Date: August 24, 2018

Atty. Docket: 4140.01500B1

Title: **ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND
METHODS OF USE THEREOF**

Amendment and Reply Under 37 C.F.R. § 1.111

Mail Stop Amendment

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

In reply to the Office Action dated December 28, 2018, Applicant submits the following
Amendment and Remarks.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of
this paper.

Remarks and Arguments begin on page 3 of this paper.

It is not believed that extensions of time are required beyond those that may otherwise be
provided for in documents accompanying this paper. However, if additional extensions of time are
necessary to prevent abandonment of this application, then such extensions of time are hereby
petitioned under 37 C.F.R. § 1.136(a), and any additional fees required to continue prosecution or
appeal of this application (including issue fee, fees for net addition of claims or forwarding to
appeal) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Reply to Office Action of December 28, 2018

WILTON *et al.*
Application No. 16/112,453

Amendments to the Claims

This listing of claims will replace all prior versions, and listings, of claims in the application.

1. (Currently Amended) A method for treating a patient with Duchenne muscular dystrophy (DMD) in need thereof who has a mutation of the DMD gene that is amenable to exon 53 skipping, comprising administering to the patient an antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, ~~wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69),~~ wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.
2. (Previously Presented) The method of claim 2, wherein the antisense oligonucleotide is administered intravenously.

Reply to Office Action of December 28, 2018

WILTON *et al.*
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Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 1 and 2 are pending in the application, with claim 1 being the independent claim. Claim 1 is sought to be amended. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicant respectfully requests that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Rejection under 35 U.S.C. § 112, second paragraph

The Office rejects claims 1 and 2 under 35 U.S.C. § 112 (pre-AIA), second paragraph, as being indefinite. Office Action at 2-3. Applicant respectfully traverses the indefiniteness rejection.

The indefiniteness rejection is based on the Office's interpretation of the claim phrase "wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)." The Office's interpretation of this phrase is that it delineates a target region 9 bases in length, i.e., the bases from positions 47 and 39. Office Action at 2. Because the Office interprets the target region as being only 9 bases in length, the Office concludes that "it is not clear how a 20 to 31 base oligonucleotide would be 100% complementary to consecutive nucleotides of the target region." Office Action at 2-3.

Applicant respectfully disagrees with the Office's interpretation of the claims. The claim phrase the "target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)" actually delineates a target region on exon 53 that falls within two overlapping annealing sites and thus provides a target region spanning from, and including, endpoint H53A+23 to, and including,

Reply to Office Action of December 28, 2018

WILTON *et al.*
Application No. 16/112,453

endpoint H53A+69. Thus, the claimed target region has clearly defined boundaries and is large enough to have 100% complementarity to oligonucleotides from 20 to 31 bases in length. As such, the claims are not indefinite. However, solely to advance prosecution of the application, Applicant amends the claims to delete this phrase.

Accordingly, the indefiniteness rejection should be withdrawn.

Rejection under 35 U.S.C. § 112, first paragraph

The Office rejects claims 1 and 2 under 35 U.S.C. § 112 (pre-AIA), first paragraph, as lacking enablement. Office Action at 3-7. Applicant respectfully traverses the lack of enablement rejection.

As with the indefiniteness rejection discussed above, the lack of enablement rejection is based on the Office's interpretation of the claim phrase "wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)" as delineating a target region only 9 bases in length. Office Action at 4-7. Based on this claim interpretation, the Office alleges that the claims lack enablement for a method of treating Duchenne muscular dystrophy using "an antisense oligonucleotide up to 31 nucleotides in length wherein the oligonucleotide only binds within a 9 nucleotide target region." Office Action at 5.

As discussed above, the claim phrase the "target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)" actually delineates a target region on exon 53 that falls within two overlapping annealing sites and thus provides a target region spanning from, and including, endpoint H53A+23 to, and including, endpoint H53A+69. Thus, the claimed target region is large enough to have 100% complementarity to oligonucleotides from 20 to 31 bases in

Reply to Office Action of December 28, 2018

WILTON *et al.*
Application No. 16/112,453

length. As such, the claims are enabled. However, solely to advance prosecution of the application, Applicant amends the claims to delete this phrase.

The Office's enablement rejection is based on the Office's allegation that the target region delineated in the claim phrase "wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)" is only 9 bases in length. As this phrase is deleted from claim 1, the lack of enablement rejection is overcome and should be withdrawn.

Double Patenting Rejection

Claims 1 and 2 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-2 of U.S. Patent No. 9,994,851. Office Action at 8. While Applicant does not agree with the double-patenting rejection, in order to advance prosecution of the application a Terminal Disclaimer over U.S. Patent No. 9,994,851 is submitted with this response.

Claims 1 and 2 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-25 of U.S. Patent No. 8,232,384. Office Action at 8. While Applicant does not agree with the double-patenting rejection, in order to advance prosecution of the application a Terminal Disclaimer over U.S. Patent No. 8,232,384 is submitted with this response.

Claims 1 and 2 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 45-64 of U.S. Application No. 15/645,842. Office Action at 8-9. While Applicant does not agree with the double-patenting rejection, in order to

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Reply to Office Action of December 28, 2018

WILTON *et al.*
Application No. 16/112,453

advance prosecution of the application a Terminal Disclaimer over U.S. Application No. 15/645,842 is submitted with this response.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicant therefore respectfully requests that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicant believes that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

/John M. Covert, #38,759/

John M. Covert
Attorney for Applicant
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Date: January 17, 2019

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Atty. Dkt. No. 4140.01500B1

SRPT-VYDS-0006279



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NOTICE OF ALLOWANCE AND FEE(S) DUE

153767 7590 02/12/2019
 STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
 1100 NEW YORK AVENUE, N.W.
 WASHINGTON, DC 20005

EXAMINER

CHONG, KIMBERLY

ART UNIT

PAPER NUMBER

1635

DATE MAILED: 02/12/2019

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/112,453	08/24/2018	Stephen Donald WILTON	4140.01500B1	3144

TITLE OF INVENTION: ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF

APPL. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	\$500	\$0.00	\$0.00	\$500	05/13/2019

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

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If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

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By fax, send to: (571)-273-2885

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153767 7590 02/12/2019
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
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I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/112,453	08/24/2018	Stephen Donald WILTON	4140.01500B1	3144

TITLE OF INVENTION: ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF

APPL. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	\$500	\$0.00	\$0.00	\$500	05/13/2019

EXAMINER	ART UNIT	CLASS-SUBCLASS
CHONG, KIMBERLY	1635	514-04400A

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively, 1
- (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed. 2
- 3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☐ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies

4b. Method of Payment: (Please first reapply any previously paid fee shown above)

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- ☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No.

5. Change in Entity Status (from status indicated above)

- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
- ☐ Applicant asserting small entity status. See 37 CFR 1.27
- ☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

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Date

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/112,453	08/24/2018	Stephen Donald WILTON	4140.01500B1	3144
153767	7590	02/12/2019	EXAMINER	
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C. 1100 NEW YORK AVENUE, N.W. WASHINGTON, DC 20005			CHONG, KIMBERLY	
			ART UNIT	PAPER NUMBER
			1635	

DATE MAILED: 02/12/2019

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
 (Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

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The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. **DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.** Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
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9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 16/112,453	Applicant(s) WILTON et al.	
	Examiner KIMBERLY CHONG	Art Unit 1635	AIA Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to 01/17/2019.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are 1-2. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a) ☐ All b) ☐ Some *c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. _____.

3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

* Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)

2. ☒ Information Disclosure Statements (PTO/SB/08),
Paper No./Mail Date 01/17/2019.

3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.

4. ☐ Interview Summary (PTO-413),
Paper No./Mail Date. _____.

5. ☐ Examiner's Amendment/Comment

6. ☒ Examiner's Statement of Reasons for Allowance

7. ☐ Other _____.

/KIMBERLY CHONG/
Primary Examiner, Art Unit 1635

Application/Control Number: 16/112,453
Art Unit: 1635

Page 2

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first to invent provisions.

Reasons for Allowance

The following is an examiner's statement of reasons for allowance: the amendments filed 01/17/2019 have overcome the rejections of record. Claims 1 and 2 are in condition for allowance.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **KIMBERLY CHONG** at (571)272-3111. The examiner can normally be reached Monday thru Friday between M-F 8:00am-4:30pm.

If attempts to reach the examiner by telephone are unsuccessful please contact the SPE for 1674 Ram Shukla at 571-272-0735. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight

Application/Control Number: 16/112,453
Art Unit: 1635

Page 3

(EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see <http://pair-direct.uspto.gov>.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Primary Examiner
Art Unit 1635

EXHIBIT 24

Volume 7, Number 3, June 1997
ISSN 1087-2906

Antisense & Nucleic Acid Drug Development

(The Antisense Journal)

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Antisense & Nucleic Acid Drug Development

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Review Article

Morpholino Antisense Oligomers: Design, Preparation, and Properties

JAMES SUMMERTON and DWIGHT WELLER

ABSTRACT

Antisense promised major advances in treating a broad range of intractable diseases, but in recent years progress has been stymied by technical problems, most notably inadequate specificity, ineffective delivery into the proper subcellular compartment, and unpredictable activity within cells. Herein is an overview of the design, preparation, and properties of Morpholino oligos, a novel antisense structural type that solves the sequence specificity problem and provides high and predictable activity in cells. Morpholino oligos also exhibit little or no nonantisense activity, afford good water solubility, are immune to nucleases, and are designed to have low production costs.

INTRODUCTION

OLIGONUCLEOTIDES, OLIGONUCLEOTIDE ANALOGS, and other sequence-specific binding polymers designed to block translation of selected messenger RNAs (the sense strand) are commonly called "antisense oligos." Development of such oligos for therapeutic applications, which constitutes the epitome of rational drug design, entails selecting a target genetic sequence unique and critical to the pathogen or pathogenic state one wishes to treat. One then assembles an oligomer of genetic bases (adenine, cytosine, guanine, and thymine or uracil) complementary to that selected sequence. When such an antisense oligo binds to its targeted disease-causing sequence, it can inactivate that target and thereby alleviate the disease.

Antisense oligos offer the prospect of safe and effective therapeutics for a broad range of intractable diseases. Nonetheless, developing therapeutics that function by a true antisense mechanism presents a number of forbidding challenges. The oligos should achieve adequate efficacy at a concentration attainable within the cells of the patient. They should inhibit their selected target sequences without concomitant attack on any other sequences in the patient's pool of approximately 200 million bases of unique-sequence RNA. They should be stable in extracellular compartments and within cells. They must be deliverable into the cellular compartment(s) containing their targeted sequences. They should be adequately soluble in aqueous solution. They should exhibit little or no toxicity at therapeutic concentrations. Finally, they should be affordable, reflecting the in-

creasing awareness that health care, even for life-threatening conditions, should not expend an excessive portion of society's resources.

First-generation antisense oligos comprised natural genetic material (Belikova et al., 1967; Zamecnik and Stephenson, 1978; Summerton, 1979) and often contained crosslinking agents for binding their targets irreversibly (Summerton and Bartlett, 1978a,b). As the design challenges became more fully appreciated, a number of nonnatural antisense structural types (Fig. 1) were developed in an effort to improve efficacy, stability, and delivery. Of particular note are the early non-ionic DNA analogs developed by Miller and Ts'o, including phosphotriester-linked DNA (Miller, 1989a) and methylphosphonate-linked DNA (Miller, 1989b). Other nucleic acid analogs of note include carbamate-linked DNA (Stirchak et al., 1987), phosphorothioate-linked DNA (Stein and Cohen, 1989), phosphoroamidate-linked DNA (Froehler et al., 1988), α -DNA (Rayner et al., 1989), and 2'-O-methyl RNA (Shibahara et al., 1989). Figure 1B shows several novel antisense types that no longer resemble nucleic acids. These oligos contain acyclic backbone moieties, including nylon (Weller et al. 1991; Huang et al., 1991), the exceptionally high-affinity peptide nucleic acids (PNAs) (Egholm et al., 1992), and related types (Summerton and Weller, 1993a).

Although each of these newer structural types provides one or more significant advantages over the first-generation oligos, none yet appear to provide the full combination of properties needed in antisense therapeutics for clinical applications.

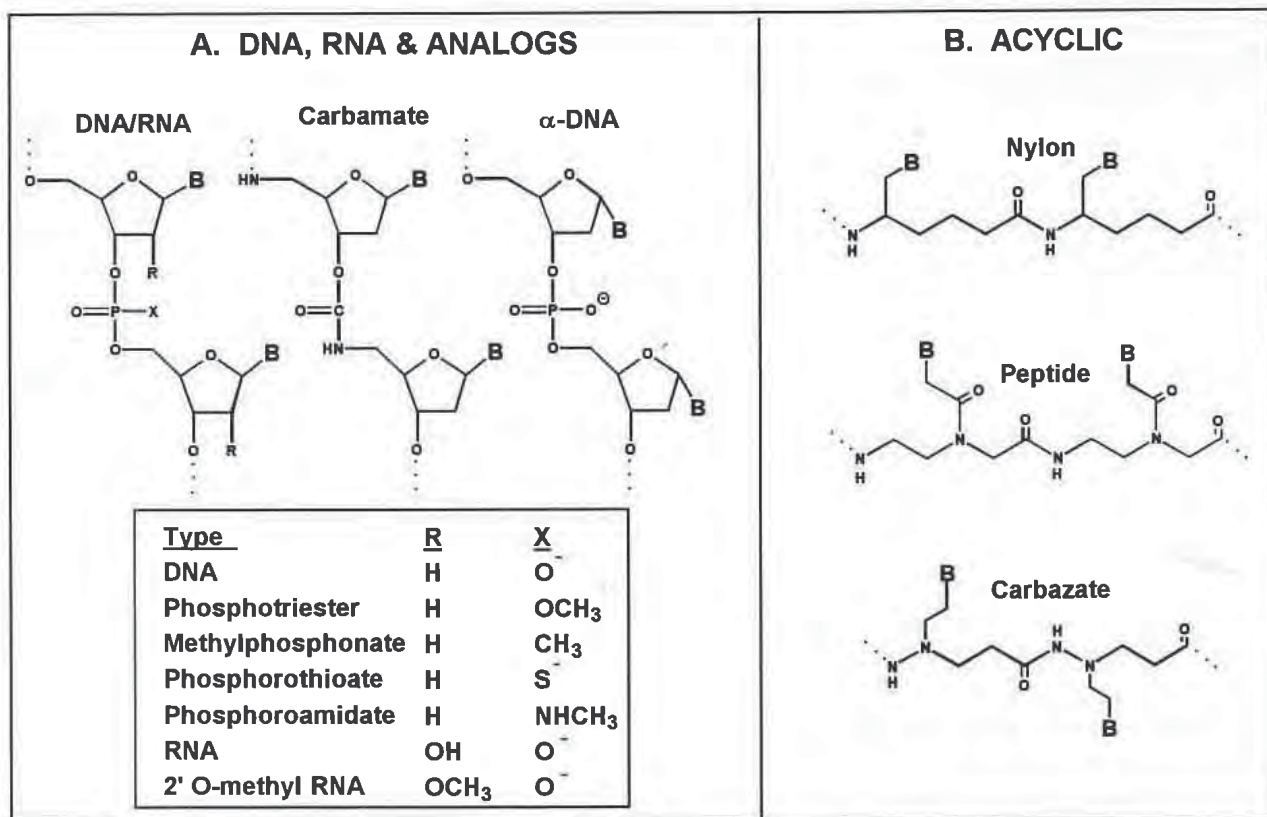


FIG. 1. Representative antisense structural types.

Herein we describe the design considerations used in developing a novel Morpholino structural type (Fig. 2), which affords antisense oligos having very high efficacy and specificity, im-

munity to nucleases, good aqueous solubility, and low production costs.

DESIGN

Backbone structure

A dominant consideration in the design of most antisense oligos has been to devise a structure that provides resistance to nucleases while still resembling natural nucleic acids as closely as possible. This conservative approach has spawned a number of DNA analogs (Fig. 1A) that may be unduly expensive for routine applications requiring systemic delivery. The high cost of DNA and its analogs is due in part to the low abundance of DNA in production-scale source material and the difficulty in cleaving DNA to the deoxyribonucleosides required for preparing DNA analogs. An additional factor in their high cost is the complexity and expense of coupling to hydroxyls, required in forming the phosphoester intersubunit linkages of most DNA analogs.

Rather than trying to solve inherent cost problems after a structural type has been developed, a better approach is to incorporate fundamental cost advantages in the initial structural design stage. Following this strategy, we reasoned that more affordable antisense oligos might be possible if inexpensive ribonucleosides could be exploited as starting material. The order-of-magnitude cost advantage of ribonucleosides relative to deoxyribonucleosides (Summerton, 1992) derives from the six-fold greater abundance of RNA relative to DNA in production-scale source material (e.g., yeast cake) and the ease of cleaving

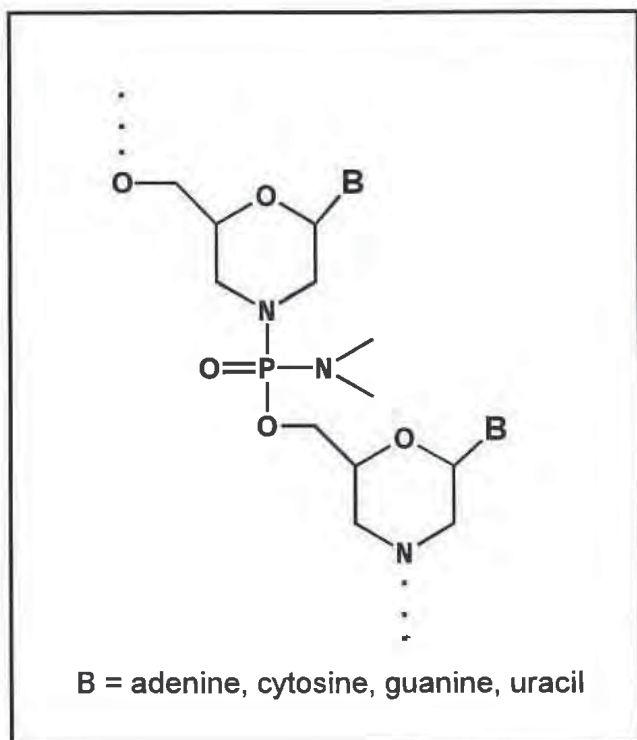


FIG. 2. Morpholino oligo structure.

RNA to its component ribonucleosides. It is noteworthy that ribonucleosides are now directly available from special excreting strains of yeast, further reducing their cost. However, the use of ribonucleosides for preparation of RNA and RNA analogs presents two serious problems. First, during oligo assembly, one must selectively couple either the 2' or the 3' hydroxyl. This is typically achieved in a relatively expensive manner by selectively masking the 2' hydroxyl with a cleavable or noncleavable moiety. The second problem is that coupling to the 3' hydroxyl of the riboside is even more difficult and expensive than the corresponding coupling of deoxyribonucleosides.

We envisioned that these problems could be circumvented by converting the riboside moiety to a morpholine moiety (Stirchak et al., 1989; Summerton, 1990) (Fig. 3). Although oligomers assembled from such Morpholino subunits differ substantially from DNA, RNA, and analogs thereof, our initial modeling studies carried out in 1985 suggested that such novel Morpholino-based oligomers might constitute useful and highly cost-effective antisense agents. The simple and inexpensive ribose to morpholine conversion shown in Figure 3 replaces two poor nucleophiles (the 2' and 3' hydroxyls) with a single good nucleophile (the morpholine nitrogen) and allows oligo assembly via simple and efficient coupling to the morpholine nitrogen without the expensive catalysts and postcoupling oxidation steps required in the production of most DNA-like antisense oligos. It is noteworthy that in spite of the relatively low nucleophilicity of the morpholine nitrogen ($pK_a = 5.75$), we still typically achieve coupling efficiencies of 99.7% without using catalysts.

Intersubunit linkage

We have assessed a substantial number of intersubunit linkage types, including the carbonyl, sulfonyl, and phosphoryl linkages (Fig. 4) (Summerton and Weller, 1991, 1993a,b; Stirchak et al., 1989). Although Morpholino oligos containing a number of such linkages provide effective binding to targeted genetic sequences, consideration of cost and ease of synthesis, chemical stability, aqueous solubility, and affinity and homogeneity of binding to RNA led us to focus on the phosphorodiamidate shown in Figure 2 as our principle linkage type for oligos targeted against single-stranded RNA sequences. These non-ionic phosphorodiamidate-linked Morpholino oligos exhibit quite good binding to complementary nucleic acids, particularly RNA sequences. Table 1 compares the temperature of melting (T_m) values at physiologic salt concentration for identical-sequence 20-mer oligos of three different antisense structural types paired with their complementary RNA. As seen in Table 1, RNA binding affinity is lowest for the phosphoroth-

ioate-linked DNA (S-DNA), appreciably higher for DNA, and highest for the Morpholino oligo.

PREPARATION

Oligo assembly

Although phosphorodiamidate-linked Morpholino oligos can be assembled by a variety of methods, one relatively simple method that has proved effective (Summerton and Weller, 1993b) entails protection and activation of the Morpholino subunit (Fig. 5A). The activated subunits can be stored at low temperatures for many months without significant breakdown. Whereas they are relatively resistant to hydrolysis, they react rapidly ($T_{1/2}$ of 1–2 minutes) with the morpholine nitrogen of growing chains on a 1% crosslinked polystyrene synthesis support loaded at 500 $\mu\text{M/g}$ of resin, with coupling efficiencies typically about 99.7%. A preferred oligo assembly cycle (Summerton and Weller, 1993b) is shown in Figure 5B. It is noteworthy that in large-scale syntheses, excess activated subunit used in the coupling step can be recovered and reused, effecting a further substantial reduction in production costs.

Because of cheaper starting materials and simpler, more efficient oligo assembly, we estimate that in large-scale production, the cost of these Morpholino antisense oligos will be at least an order of magnitude lower than the cost of corresponding DNA analogs (Summerton, 1992).

PROPERTIES

Solubility

For an antisense oligo to have effective access to its target sequence within the cytoplasm of a cell, the oligo should show reasonable water solubility. Good water solubility may also prove essential for systemic delivery of antisense oligos. Conventional wisdom in the antisense field is that non-ionic antisense oligos invariably show poor water solubility. In this regard, it is interesting that a Morpholino dimer containing a rigid carbamate linkage shows little or no base stacking (Kang et al., 1992), and in the absence of special solubilizing groups, Morpholino oligomers containing such carbamate linkages are quite insoluble in aqueous solutions (Stirchak et al., 1989). In contrast, phosphorodiamidate-linked Morpholino oligos of the type shown in Figure 2 show excellent base stacking (Kang et al., 1992) and are several orders of magnitude more soluble in aqueous solutions. To illustrate the exceptional aqueous solu-

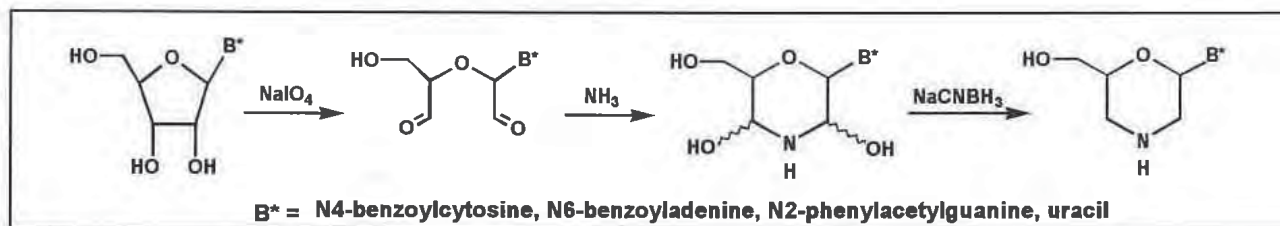


FIG. 3. Conversion of ribonucleoside to Morpholino subunit.

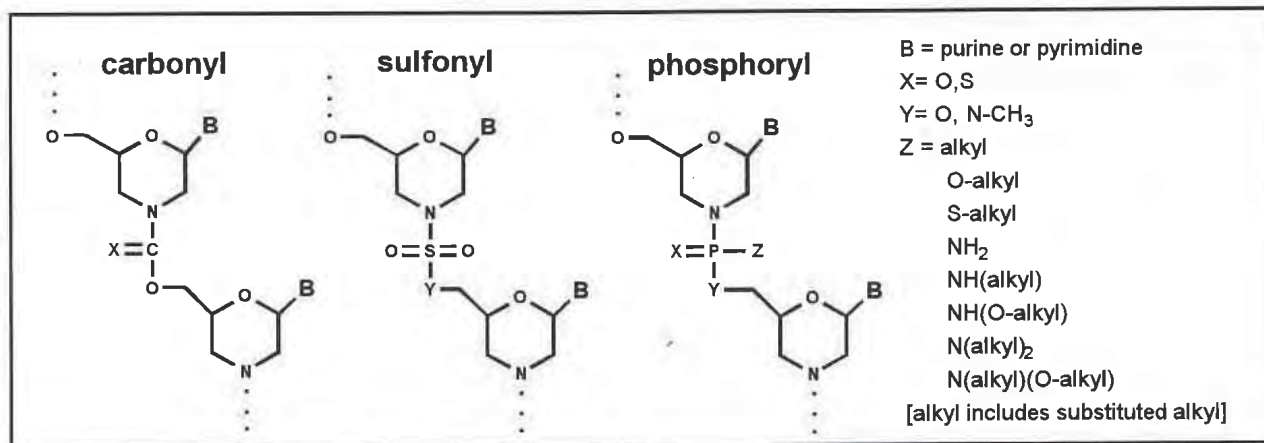


FIG. 4. Intersubunit linkage types for Morpholino oligos.

bility of Morpholino oligos of this type, we have dissolved 263 mg of a heteromeric 22-mer of the sequence 5'-GCUCGCA-GACUUGUCCAUCAU in 1 ml of water (36 millimolar) at 20°C without reaching saturation.

We suggest that the poor water solubility of the carbamate-linked Morpholino oligos results at least in part from the difficulty of inserting the hydrophobic faces of the unstacked bases into an aqueous environment. In contrast, it seems likely that the excellent water solubility of the phosphorodiamidate-linked Morpholino oligos is a consequence of effective shielding of these hydrophobic faces from the polar solvent because of good stacking of the bases.

Biologic stability

To achieve reasonable efficacy, an antisense oligo should not be degraded rapidly either extracellularly or within cells. In this regard, it has been demonstrated that DNA and 2'-O-methyl RNA are rapidly degraded and phosphorothioate DNA is slowly degraded by nucleases in blood and within cells (Hoke et al., 1991; Morvan et al., 1993). Although resistance to nucleolytic degradation can be improved by adding special groups to the termini (Cazenave et al., 1987) or by incorporating a few nuclease-resistant intersubunit linkages near each end (Larrouy et al., 1992), we believe a better solution, on the basis of both function and cost, is to use a backbone structure that is inherently immune to a broad range of degradative enzymes present in the blood and within cells. A further advantage of using a backbone structure that is not degraded in the body is that it avoids concerns that modified nucleosides or nucleotides resulting from degradation of an antisense oligo might be toxic or might be incorporated into cellular genetic material and thereby lead to mutations or other undesired biologic effects.

In experiments detailed elsewhere (Hudziak et al., 1996), it is demonstrated that Morpholino phosphorodiamidate oligos of

the type shown in Figure 2 are immune to a wide range of nucleases, including DNase I (an endonuclease that cleaves both single-stranded and double-stranded DNA), DNase II (cleaves between the 5' oxygen and the phosphorus of DNA linkages), RNase A (cleaves on the 3' side of pyrimidines), RNase T1 (cleaves on the 3' side of guanines), nuclease P1 (cleaves single-stranded RNA and DNA), phosphodiesterase (3' exonuclease for both RNA and DNA), Mung bean nuclease (cleaves single-stranded RNA and DNA), and benzonase (cleaves both single-stranded and double-stranded RNA and DNA, including linear, circular, and supercoiled). These Morpholino oligos have also been found to be immune to pronase E, proteinase K, and pig liver esterase, as well as degradative enzymes in serum and a liver homogenate.

Antisense efficacy

Because of the excellent RNA binding affinity of oligos of this phosphorodiamidate-linked Morpholino structural type, it seemed likely Morpholino oligos would be effective in blocking translation of their targeted mRNAs, and this has been found to be the case. In cell-free translation experiments using a sensitive luciferase reporter, we have demonstrated that a Morpholino oligo 25 subunits in length, in both the presence and absence of RNase H, inhibits its targeted mRNA somewhat better than the corresponding S-DNA oligo in the presence of added RNase H, with both showing good efficacy at concentrations of 10 nM and above. Representative translational inhibition results are shown in Figure 6 (Summerton et al., 1997). A similar comparison of Morpholino and S-DNA antisense oligos targeted against murine tumor necrosis factor- α (TNF- α) mRNA in a cell-free translation system also showed greater activity for the Morpholino oligos (Taylor et al., 1996).

Specificity

In the early days of antisense research, one of the most compelling arguments for antisense therapeutics was their promise of exquisite specificity for their targeted genetic sequences. However, as the most synthetically accessible antisense structural types (DNA and S-DNA) have come into broad use, it has become clear that these two structural types provide reasonable

TABLE 1. MELTING TEMPERATURES OF RNA/OLIGO DUPLEXES

RNA/S-DNA	68.5°C
RNA/DNA	77.3°C
RNA/Morpholino	81.3°C

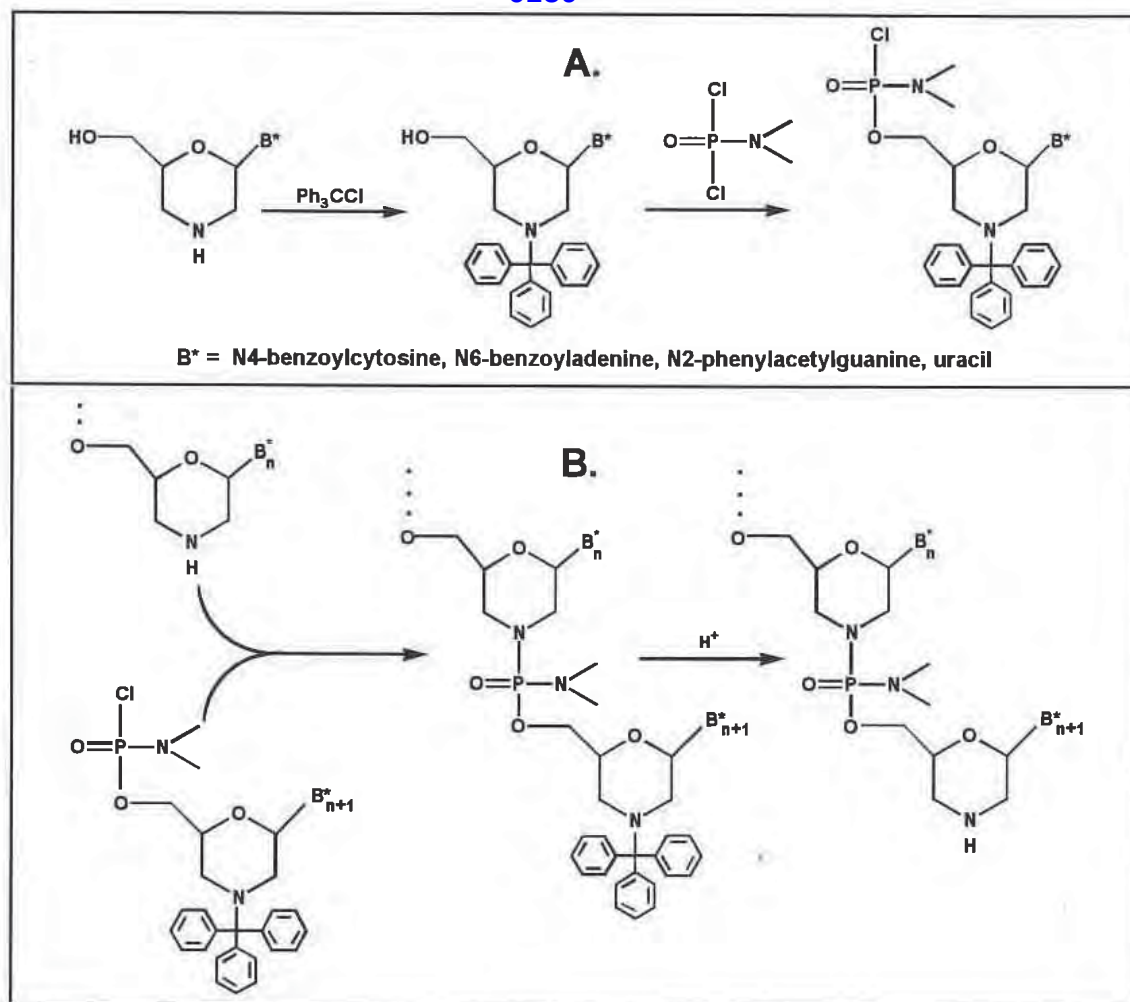


FIG. 5. Protection, activation, and coupling of Morpholino subunits.

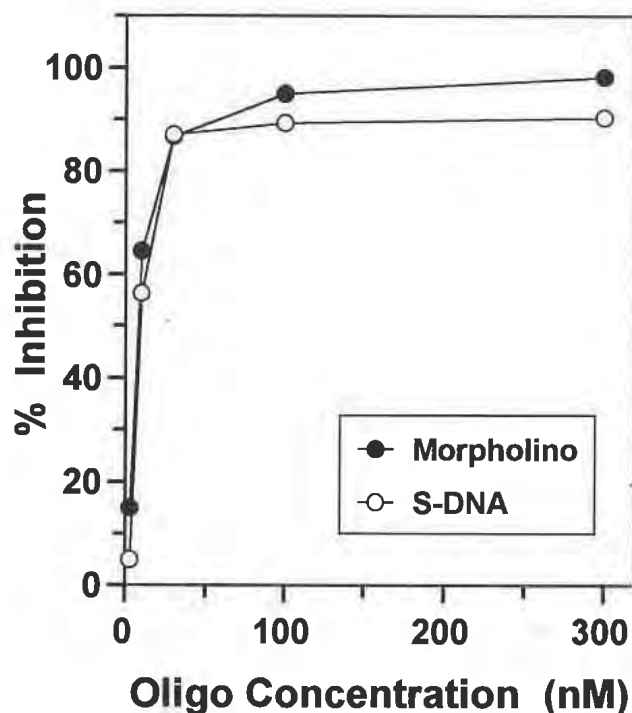


FIG. 6. Cell-free efficacy of Morpholino and S-DNA antisense oligos.

sequence specificity within only a very narrow concentration range (ANTIVIRALS Inc., 1993; Stein and Cheng, 1993).

We believe a key factor responsible for the low specificity of DNA and S-DNA oligos is their RNase H competency; that is, DNA and S-DNA form duplexes with complementary RNA that are readily cleaved by RNase H, an enzyme widely distributed in living organisms. The specificity problem arises because DNA/RNA and S-DNA/RNA duplexes as short as 5 base pairs in length are cleaved by RNase H (Crouch and Dirksen, 1982). Presuming about 6% of the genome is transcribed in higher animals, the patient's RNA pool will comprise about 200 million bases of unique-sequence RNA. With this level of sequence complexity, it is inevitable that antisense oligos will form many short transient duplexes with partially complementary nontarget sequences of inherent cellular RNAs. Cleavage of the RNA strand of such nontarget duplexes by endogenous RNase H (Larrouy et al., 1992; Cazenave et al., 1989) is expected to cause significant disruption of normal cellular translation. As this cleavage process releases the DNA or S-DNA in its original form, such oligos can continue the cycle of transiently pairing with additional nontarget cellular RNA sequences, cleavage of the RNA strand, and release of the antisense oligo. As a consequence, essentially every RNase H-competent oligo is expected to cleave hundreds to thousands of species of inherent cellular RNAs.

A second factor expected to contribute to superior specificity of Morpholino oligos relative to RNase H-competent types is that RNase H-independent oligos have far fewer potential targets in the inherent pool of cellular RNA. This is because most antisense structural types that do not support RNase H cleavage of their RNA targets have been found to be effective in blocking translation of their targeted mRNAs only when said oligos are complementary to sequences in the 5' leader region of that mRNA or when they are targeted against other special sites, such as splice junctions and transport signals [e.g., methylphosphonate DNA (Walder and Walder, 1988), α -DNA (Rayner et al., 1989), 2'-O-methyl RNA (Shibahara et al., 1989), and Morpholino (Summerton et al., 1997)]. We estimate that such special targetable regions constitute on the order of 2%–5% of the sequences in the cellular RNA pool. Presumably, this targeting limitation reflects the ability of ribosomes to displace essentially all antisense oligos during translocation down the coding region of mRNAs.

Because an antisense oligo that does not support RNase H cleavage cannot effectively block functioning of an RNA when said oligo is bound to sequences outside of special targetable regions, such an oligo only needs to distinguish its target sequence from those 2%–5% of the cellular RNA sequences comprising special targetable regions. In contrast, antisense oligos that form RNase H-cleavable duplexes with RNA can be effective when targeted essentially anywhere along an RNA transcript (Walder and Walder, 1988), presumably because RNase H cleavage at the target site of the antisense oligomer destroys the RNA, rendering moot the oligo displacement capability of translocating ribosomes. Accordingly, RNase H-competent oligos (DNA and S-DNA) face the much greater specificity challenge of distinguishing selected target sequences from essentially the entire pool of cellular RNA sequences. As a consequence, RNase H-independent oligos, such as Morpholinos, should enjoy a 20-fold to 50-fold advantage in sequence

specificity because of this more than order-of-magnitude reduction in the number of inherent nontarget cellular sequences of any given length that they can inhibit.

A third factor compromising the specificity of S-DNA oligos is their promiscuous binding to proteins (Krieg and Stein, 1995), including components of the cell's replication, transcription, and translation machinery.

Given these factors expected to limit the sequence specificity of RNase H-competent antisense structural types, particularly S-DNA, we set out to compare sequence specificities of S-DNA and Morpholino antisense oligos. To this end, we carried out stringent specificity assays in a cell-free translation system using two oligos of each structural type (Summerton et al., 1997). In these experiments, one oligo was perfectly complementary to its target mRNA to provide a measure of the total inhibition afforded by that oligo type. The other oligo incorporated 4 mispairs to that same mRNA target sequence, with the longest run of perfect pairing comprising 10 contiguous base pairs, to provide an estimate of the low-specificity component of the inhibition. The difference between these two inhibition values at each concentration then provided a measure of the high-specificity component, which we denote as "sequence-specific inhibition."

Figure 7 (experimental as in Summerton et al., 1997) shows that the S-DNA oligo achieved reasonable efficacy at concentrations above about 10 nM, but the sequence-specific component of its inhibition dropped below 50% at concentrations of only 100 nM and higher. The corresponding Morpholino oligo achieved even better efficacy at 10 nM while maintaining good sequence specificity through 10,000 nM, the highest concentration tested. Thus, in this stringent test of specificity, the Morpholino oligo achieved highly effective and specific antisense activity over a concentration range more than two orders of magnitude greater than the concentration range wherein the corresponding S-DNA achieved reasonable efficacy and specificity.

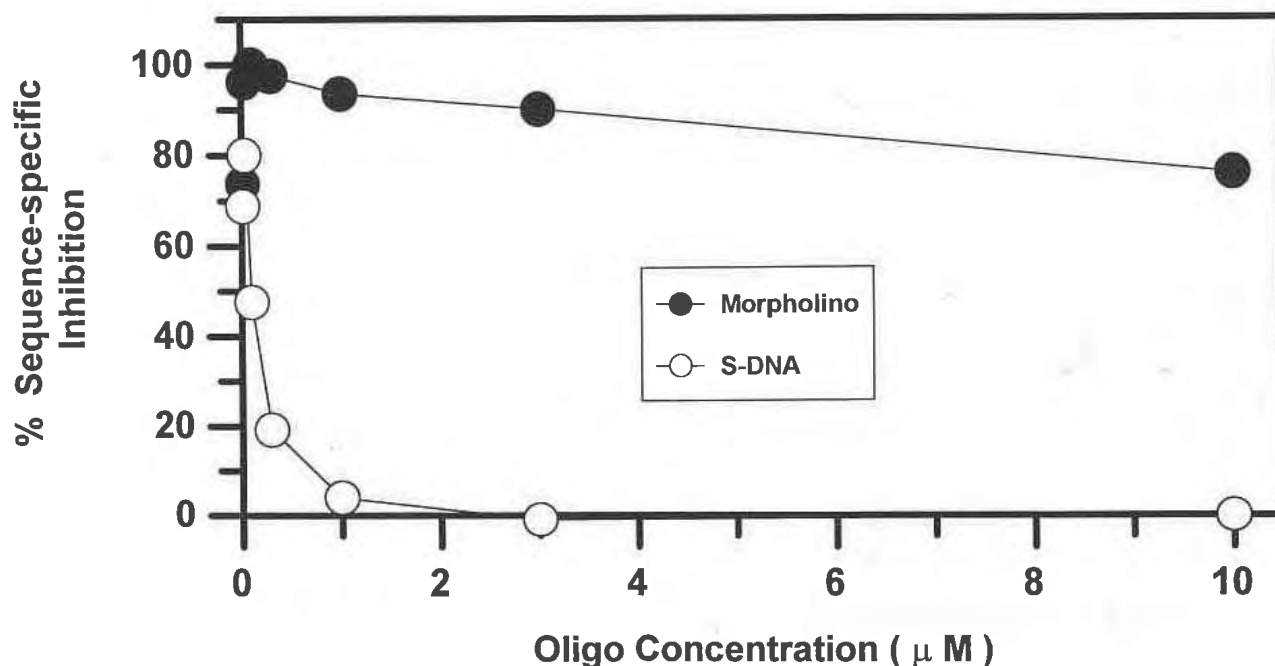


FIG. 7. Sequence specificity of Morpholino and S-DNA oligos.

Taylor et al., (1996) have reported that S-DNAs targeted against TNF- α mRNA showed very poor sequence specificity in a cell-free translation system, whereas the corresponding Morpholino oligos afforded good specificity over the full range tested.

Activity in cells

For effective biologic activity, an antisense oligo must gain entry into the cellular compartments where the target genetic sequence is synthesized, processed, and functions—specifically, the cytosol/nuclear compartment. Our experiments with fluorescent-tagged Morpholino oligos suggested that these oligos enter mammalian cells by what appears to be endocytosis, but they do not appear to subsequently cross the endosomal or lysosomal membrane into the cytosol, based both on visualization of fluorescent-tagged oligos and a functional assay employing a transfected plasmid (Partridge et al., 1996). This result is in agreement with limitations on uptake of antisense oligos reported by others. Specifically, a number of studies have been reported that suggest that in the absence of experimental manipulations that compromise the cell membrane, both polyanionic oligos [e.g., S-DNA (Wagner et al., 1993; Tonkinson and Stein, 1994) and 2'-O-methyl RNA (Oberhauser and Wagner, 1992)] and non-ionic oligos [e.g., methylphosphonate DNA (Shoji et al., 1991) and PNAs (Bonham et al., 1995)] enter cells primarily or exclusively by endocytosis. Further, a number of studies on a variety of antisense structural types indicate that most or all of the antisense oligo that gains entry by endocytosis does not subsequently traverse

the endosomal or lysosomal membrane to enter in an intact form into the cytosol, where protein synthesis occurs (Oberhauser and Wagner, 1992; Shoji et al., 1991; Bonham et al., 1995).

However, we have found that antisense oligos can be easily delivered into cultured cells simply by passaging anchorage-dependent cells by the common procedure of scraping with a rubber policeman. This has been shown to achieve significant oligo entry into the cytosolic compartment if the oligo is present during the scraping (Partridge et al., 1996). Further, Morpholino oligos delivered into cells by such scrape loading show good activity and specificity therein, whereas corresponding S-DNA oligos (both antisense and control sequences) largely fail to inhibit their targets within scrape-loaded cells at concentrations up to 3 μ M in the medium and instead are often stimulatory (Summerton et al., 1997). Figure 8 shows a comparison of the activities of representative Morpholino and S-DNA oligos in scrape-loaded cells (experimental as in Summerton et al., 1997).

Taylor et al. (1996) have also compared the activity of S-DNA and Morpholino antisense oligos in cultured cells. In their studies, the S-DNAs were delivered into mouse macrophage-like cells (RAW 264.7) using lipofectin. Both oligo types were targeted against TNF- α mRNA, and treated cells were assessed for inhibition of lipopolysaccharide-induced TNF- α production. In agreement with our in-cell results, Taylor et al. report that both the antisense and control S-DNAs stimulated instead of inhibited TNF- α production, whereas the Morpholino antisense oligo, although poorly delivered into the cells, afforded significant and specific inhibition of TNF- α production.

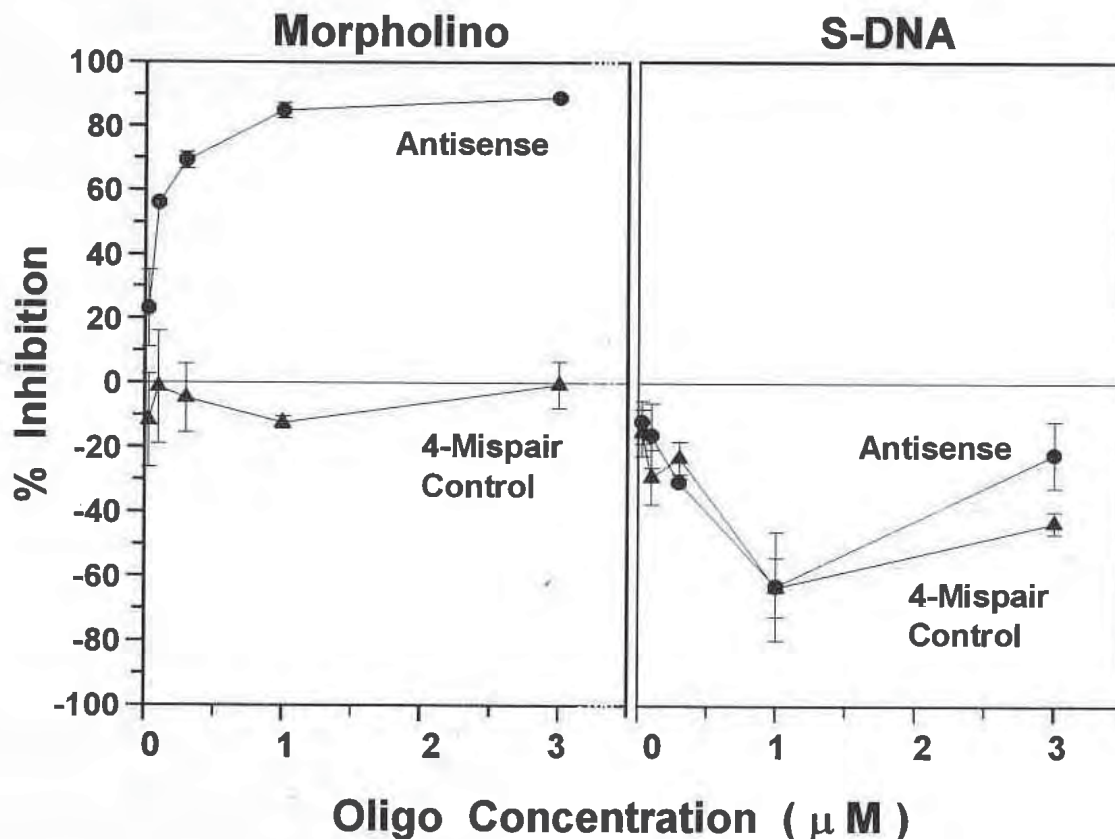


FIG. 8. In-cell activities of Morpholino and S-DNA oligos.

In vivo properties

To date, our principal efforts have focused on optimizing the Morpholino structural type and on studying the properties of Morpholino oligos at the biophysical level, in cell-free translation systems, and in cultured cells. In light of the promising results from those studies, we and several collaborators are now shifting our focus to *in vivo* studies.

A very preliminary ranging study was carried out to assess acute toxicity. In this study, a representative 20-mer Morpholino oligo in phosphate-buffered saline was injected intravenously into mice at doses ranging from 88 mg/kg to 700 mg/kg. No acute toxicity was seen at any of these doses. However, over a period of 2 weeks, an effect on body weight gain and ruffled coat was observed at the highest dose. Using the results from this ranging study, an extensive toxicity study has been initiated and will be the subject of a future report.

In addition, a variety of efficacy studies in mice and rats are in progress to assess the possible use of Morpholino oligos for therapeutic applications. We are also investigating possible methods for improving the delivery of these oligos into the cytosol/nuclear compartment of cells *in vivo*.

DISCUSSION

Morpholino oligos meet key requirements for safe, effective, and affordable antisense therapeutics, including high efficacy at low nanomolar concentrations, high sequence specificity over a thousandfold concentration range, little or no nonantisense activity, total stability in blood and within cells, excellent water solubility, and low production costs relative to other antisense structural types. Our efforts are now focused on achieving effective delivery into the cytosol/nuclear compartment of cells by means suitable for therapeutic applications and on studying the activities of these oligos in animals.

ACKNOWLEDGMENTS

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EXHIBIT 25

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Morpholino and Phosphorothioate Antisense Oligomers Compared in Cell-Free and In-Cell Systems

JAMES SUMMERTON, DAVID STEIN, SUNG BEN HUANG, PAULA MATTHEWS, DWIGHT WELLER,
and MICHAEL PARTRIDGE

ABSTRACT

Morpholino and phosphorothioate (S-DNA) antisense oligos were compared in both cell-free and in-cell translation systems. In the most stringent test of specificity in the cell-free system, a globin-targeted S-DNA oligo was found to inhibit its target sequence at concentrations of 10 nM and above, but the sequence-specific component of this inhibition dropped below 50% at concentrations of 100 nM and above. A corresponding Morpholino oligo achieved even higher inhibition at 10 nM, but in contrast to the S-DNA, with the Morpholino, the sequence-specific component of this inhibition remained above 93% at a concentration of 3000 nM. In this same cell-free test system, several S-DNA oligos exhibited substantial undesired nonantisense effects at concentrations of 300 nM and above, whereas corresponding Morpholino oligos exhibited little or no nonantisense activity through a concentration of 3000 nM. In scrape-loaded HeLa cells, both globin-targeted and HBV-targeted S-DNAs (both antisense and control oligos) generally failed to achieve significant translational inhibition at extracellular concentrations up to 3000 nM. In contrast, the Morpholino oligos achieved effective and specific translational inhibition at extracellular concentrations ranging from 30 nM to 3000 nM.

INTRODUCTION

SEQUENCE-SPECIFIC BINDING OLIGOMERS designed to inactivate selected messenger RNAs (the sense strands) are commonly called "antisense" oligos. Such antisense oligos hold promise as tools for studying the function of genes, as well as the prospect of safe and effective therapeutics for a wide variety of diseases. First-generation antisense oligos, comprising natural genetic material (Zamecnik and Stephenson, 1978), often with added crosslinking moieties for irreversibly binding their targeted genetic sequences (Belikova et al., 1967; Summerton and Bartlett, 1978a,b; Summerton, 1979), were found to be rapidly degraded in biologic systems. Methylphosphonate-linked DNA oligos (MP-DNAs) (Miller, 1989) were the first to provide resistance to enzymatic degradation but were found to afford limited efficacy and poor aqueous solubility. In an effort to overcome such limitations, a number of other structural types have been developed over the past decade (Crooke and Lebleu, 1993). Of these, phosphorothioate-linked DNA oligos (SDNAs, Fig. 1) have come to dominate the antisense field, being easily prepared and moderately resistant to enzymatic degradation and providing higher efficacies and much better aqueous solubility than MP-DNAs (Stein and Cohen, 1989). However, with their

widespread use, it is becoming apparent that S-DNAs provide reasonable sequence specificity only within a narrow concentration range (Stein and Cheng, 1993), and they generate a plethora of nonantisense effects, due at least in part to interactions with extracellular and cellular proteins (Jansen et al., 1995; Krieg et al., 1995; Yaswen et al., 1993; Perez et al., 1994-1 Krieg and Stein, 1995; Stein, 1995). With the goal of overcoming these remaining limitations, we have developed Morpholino oligos (Fig. 1), a novel structural type that contains six-membered morpholine backbone moieties joined by non-ionic phosphorodiamidate intersubunit linkages (Summerton and Weller, 1993; Partridge et al., 1996; Hudziak et al., 1996).

MATERIALS AND METHODS

mRNAs, plasmids, and oligos

In these studies, two messenger RNAs (mRNAs) were used, one comprising the 5' leader sequence of rabbit α -globin mRNA joined to the amino acid-coding sequence of luciferase

and the other comprising a highly conserved portion of the 5' leader sequence of the hepatitis B virus (HBV) 3.5 kb mRNA joined to the coding sequence of luciferase. For the cell-free studies, the globin and HBV mRNA constructs were transcribed from plasmids pAVI-1 and pAVI-2, respectively, containing the T7 promoter. The plasmids were linearized with a restriction nuclease and transcribed with T7 polymerase following the protocol in Ambion mMessage mMachine Instruction Manual (catalog 1344, Ambion Inc., Austin, TX). For in-cell studies, the globin and HBV-containing mRNA constructs were transcribed from plasmids pAVI-3 and pAVI-4, respectively, stably transfected into HeLa cells, with both plasmids containing the mouse mammary tumor virus promoter inducible by dexamethasone.

Figure 2 shows the 5' regions of these two mRNA constructs and corresponding antisense and control oligos used in these experiments.

S-DNA oligos having the structure shown in Figure 1 and the sequences shown in Figure 2 were purchased from Biosource International, Keystone Division (Camarillo, CA). Corresponding Morpholino oligos were synthesized at ANTIVIRALS Inc. by methods similar to those detailed elsewhere (Summerton and Weller, 1993). (Morpholino oligos and the plasmids used in these studies are commercially available from ANTIVIRALS Inc., 4575 S.W. Research Way, Corvallis, OR 97333.)

Cell-free assays

Cell-free translation reactions, run in triplicate, were modeled after those in Novagen protocol TB012. For each translation reaction, 2 µl of oligo in H₂O was added to 6 µl of mRNA solution containing 3 µl rabbit reticulocyte lysate without methionine (Novagen catalog 69360), 0.25 µl RNasin (Promega catalog N2512), 0.5 µl translation mix without methionine (Novagen catalog 69360), 0.275 µl 2.5 M KCl, 0.125 µl 25 mM magnesium acetate, 0.85 µl of H₂O (for reactions without RNase H) or RNase H solution (0.235 U/µl from US Biochemical (catalog 70054), and 1.0 µl mRNA construct in H₂O. This mixture was incubated for 60 minutes in a 37 °C air incubator (to preclude evaporation and recondensation on the walls of the tube). To start the translation process, 16 µl of lysate solution was added, comprising 11.25 µl Promega nuclease-treated reticulocyte lysate (catalog L4970), 3.75 µl H₂O, and 1.0 µl 1 mM amino acid mix (Promega catalog L4461). Oligo concentrations indicated in Figures 3, 4, 5, and 6a refer to concentrations in these final translation reaction mixes. Each translation reaction was incubated for 90 minutes at 37 °C and then chilled on ice. Quantitation of the luciferase activity generated in the translation reaction entailed adding 10 µl of the chilled preparation to 50 µl of ambient-temperature Promega luciferase assay reagent (catalog 1483), mixing 30 seconds, and measuring the light emission for 15 seconds in a Turner Model TD-20e luminometer (Turner Designs, Inc., Mountain View, CA). Separate experiments showed that in this translation mixture and in the luciferase assay solution, neither S-DNA nor Morpholino oligos at concentrations ranging from 10 nM to 10,000 nM have a significant effect on the activity of preformed luciferase.

Percent inhibition of luciferase activity values was calculated as

$$\% \text{ Inhibition} = 100(1 - (+\text{oligo}/-\text{oligo}))$$

where +oligo is the light emitted from a preparation treated with oligo and -oligo is the average of the light emitted from three control preparations not treated with oligo.

In-cell assays

In-cell inhibition assays in cells scraped in the presence of Morpholino oligos were carried out in triplicate as described in Partridge et al. (1996). Corresponding assays with S-DNAs were modified such that at the end of the posttreatment 16-hour incubation period, both free-floating and readhered cells were collected, washed three times by gentle centrifugation, and assessed for luciferase and total protein, as described in Partridge et al. (1996). The modified assay for cells scrape-loaded with SDNA oligos was necessary because a substantial fraction of cells treated with 300 nM or greater concentration of S-DNA oligo (either antisense or control) during or following scraping fails to readhere to the culture plate in the subsequent 16-hour posttreatment incubation. In this regard, we have found that when the S-DNA oligo is added a few minutes after scraping [so as to preclude oligo entry into the cytosol/nuclear compartment, which occurs predominantly within the first minute after scraping (Partridge et al., 1996)], the nonadherent cells undergo normal levels of induction, transcription, and translation of the plasmid-coded mRNAs. Further, if the S-DNA oligo is removed from the medium after the scrape-load procedure, the SDNA-treated cells readhere normally. Based on these findings, we postulate that lack of readherence in the presence of S-DNA may simply be because of the S-DNA blocking sites on the cell surface or culture plate or both required for cell readherence.

In-cell inhibition assays using S-DNA complexed with LIPOFECTINTM were modeled after procedures provided by Life Technologies Inc., from which the LIPOFECTINTM was purchased. This entailed seeding 2 X 10⁵ cells per 60-mm well and incubating in growth medium (containing 10% serum and antibiotics) for 24 hours. LIPOFECTINTM reagent (20 µl) (1 mg/ml) (Life Technologies, Inc., Catalog 18292-011) was diluted into 80 µl serum-free medium (without antibiotics) and allowed to stand 40 minutes at ambient temperature, after which it was mixed with 100 µl serum-free medium (without antibiotics) containing the S-DNA antisense oligo. After 15 minutes at ambient temperature, another 1.8 ml of serum-free medium was added to the S-DNA/LIPOFECTINTM solution. The growth medium was removed from the cells, and the cells were washed with serum-free medium (without antibiotics). Then the S-DNA/LIPOFECTINTM solution was added, and the cells were incubated for 6 hours at 37 °C. The S-DNA/LIPOFECTINTM solution was removed and replaced with growth medium containing 10% serum, antibiotics, and dexamethasone (1 µl), and incubation continued for 16 hours. Cells were then assessed for luciferase activity.

As described in Partridge et al. (1996), quantitation of Inciferase activity in samples from the in-cell experiments entailed both measurement of light emission from a sample and measurement of total protein in that sample. The light emission value was then divided by the total protein value to give a normalized light emission value. These normalized light emission values correct for sample to sample variations in the cell scrap-

TABLE 1. THERMAL MELT VALUES
FOR OLIGO/RNA DUPLEXES

	S-DNA/RNA	Morpholino/RNA
0-mismatch oligo	70°C	86°C
4-mismatch oligo	46°C	59°C

ing and transfer steps. Percent inhibition of luciferase activity was calculated as in the cell-free experiments using these normalized light emission values.

RESULTS

Cell-free studies

To illustrate the relative RNA binding affinities of these two structural types, Table 1 gives the thermal melt (T_m) values for the α -globin S-DNA and Morpholino antisense oligos of Figure 2 paired to complementary target RNA, as well as the corresponding four-mismatched control oligos paired with the same target RNA. These T_m values were obtained with oligo and target RNA each at 5 μ M in salinephosphate buffer (0.15 M NaCl, 0.05 M NaH_2PO_4 NaOH to pH 7.2).

Initial studies were carried out in a simple reticulocyte lysate translation system to provide an assessment of translational inhibition independent of the complexities associated with cell permeability barriers and subcellular compartmentation. Relative efficacies of these two structural types were assessed in both the absence and presence of added RNase H using perfectly paired antisense oligos at concentrations ranging from 10 nM to 3000 nM, with their respective target mRNAs at a concentration of 1 nM. Figure 3a shows efficacies against a globin target in the absence of added RNase H, Figure 3b shows efficacies against this same globin target in the presence of added RNase H, and Figure 3c shows efficacies against an HBV target in the presence of added RNase H.

Antisense therapeutics that lack adequate sequence specificity are expected to inhibit translation of inherent cellular mRNAs, which could be manifested as toxicity for patients. Based on an estimated cellular RNA pool complexity of approximately 200 million bases of unique-sequence RNA, we estimate that for a typical 25-base viral genetic sequence (not recently plagiarized from the host) one expects to find at least four mismatches relative to any given sequence in the inherent pool of cellular RNAs in humans. Thus, the globin four-mismatched control oligo of Figure 2 is expected to provide a reasonable emulation of the closest match a viral-targeted oligo is likely to have with any cellular RNAs of a patient. To provide a stringent assessment of relative sequence specificities of the two structural types, we used the globin zero-mismatch oligo to provide a measure of the total inhibition afforded by that oligo type and the corresponding globin four-mismatch oligo (containing a 10-contiguous-base match to the target sequence) to provide an estimate of the low-specificity component of the inhibition that could cause toxicity in patients. The difference between these two values provides a measure of the high-specificity component, which we denote as "sequence-specific inhibition" (Fig. 4a). Results from a

similar but somewhat less stringent specificity study using the HBV four-mismatch oligo (containing a 7-contiguous-base match to the target sequence) are shown in Figure 4b.

Because S-DNAs have been reported to bind a variety of proteins (Krieg and Stein, 1995) and may affect translation via such binding, it is also desirable to estimate the fraction of translational inhibition that is largely unrelated to pairing between the oligo and its target RNA. To this end, oligos having negligible complementarity to the target mRNAs were assessed for their inhibitory activity. This inhibition, denoted as "nonantisense inhibition," is shown in Figure 5.

In-cell studies

A growing body of evidence, including our fluorescence microscopy studies, suggests that in the absence of special delivery reagents, most or all antisense oligos enter unperturbed cultured animal cells predominantly or solely via endocytosis and are subsequently exocytosed or sequestered (or both) in lysosomes, with little or no intact oligo entering the cytosol/nuclear compartment (Wagner et al., 1993). However, our fluorescence microscopy studies with fluorescein-tagged oligos also indicate that both S-DNA and Morpholino oligos can be delivered rapidly and efficiently into the cytosol of adherent cells in culture by a simple scrape-load procedure (Partridge et al., 1996; unpublished observations). These studies further show that in a typical scrape-load procedure, both S-DNA and Morpholino oligos appear to enter the cytosol/nuclear compartment to about the same extent, with oligos entering about 85% of the scraped cells. In this context, it is noteworthy that Farrell et al. have reported that normal smooth muscle cells of artery walls do not take up significant S-DNA, but when those cells are perturbed in the course of balloon angioplasty, a substantial amount of S-DNA is seen to enter the cytosol/nuclear compartment of the perturbed cells (Farrell et al., 1995).

In agreement with our fluorescence microscopy observations, results from functional assays (Fig. 6) suggest that both S-DNA and Morpholino oligos fail to enter the cytosol of unperturbed HeLa cells to a significant extent during a 16-hour incubation but can readily enter the cytosol via scrape-loading. Specifically, Figure 6a shows that both S-DNA and Morpholino oligos at low nanomolar concentrations are quite effective against their mRNA target sequence in a cell-free translation system. Figure 6b shows that these same oligos at a

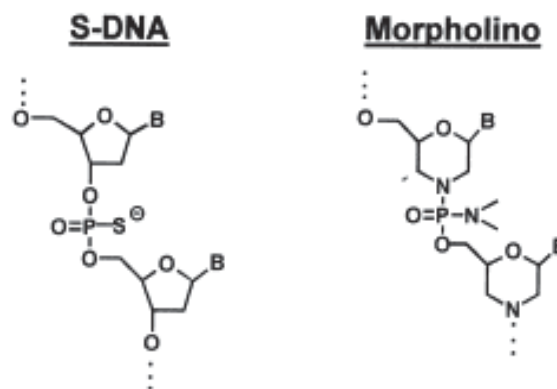


FIG. 1. Oligo structural types.

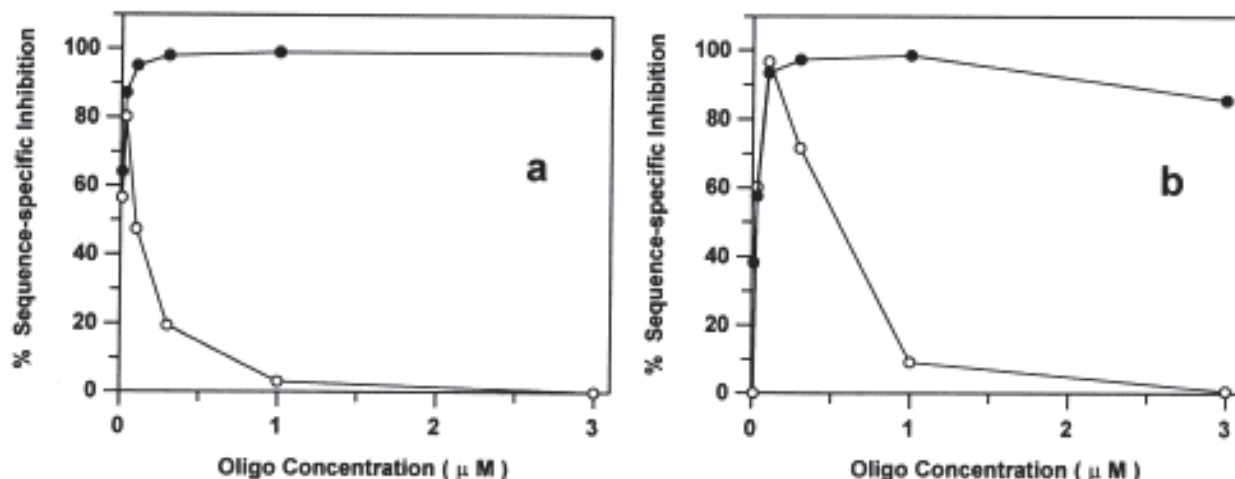


FIG. 4. Sequence-specific inhibition in cell-free system. Translation reactions, with added RNase H, were carried out with the four-mismatch control oligos, and the resulting inhibition values at each concentration were subtracted from the corresponding inhibition values for the 0-mismatch oligos to give % sequence-specific inhibition values. Any negative inhibition values (presumably due to stimulation of luciferase synthesis) were set to zero in calculating these % sequence-specific inhibition values. (a) Globin-targeted oligos/globin mRNA. (b) HBV-targeted oligos/HBV mRNA. Open circles, S-DNA; closed circles, Morpholino.

thousandfold higher concentration in the extracellular medium fail to inhibit the same mRNA target within unperturbed HeLa cells. Finally, Figure 6c demonstrates that when the Morpholino oligo is introduced into cells via scrape-loading, it is quite effective against its mRNA target therein. We used this scrape-load oligo delivery procedure to assess in HeLa cells the activities of S-DNA and Morpholino antisense oligos, with oligo concentrations ranging from 30 nM to 3000 nM in the extracellular medium.

LIPOFECTIN™ has been used to deliver S-DNAs into cultured cells (Wagner et al., 1993; Dean et al., 1994). Therefore, to provide a comparison with delivery via scrape-loading, we also -treated unperturbed HeLa cells with varying concentrations of S-DNA antisense oligo complexed with LIPO-

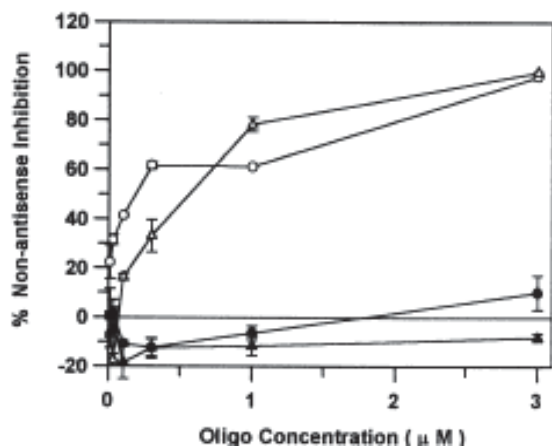


FIG. 5. Nonantisense activities in cell-free system. Translation reactions as per Figure 3, with added RNase H. Open circles, S-DNA, and closed circles, Morpholino IIBV antisense oligo and globin mRNA; open triangles, S-DNA, and closed triangles, Morpholino globin antisense oligo and HBV mRNA.

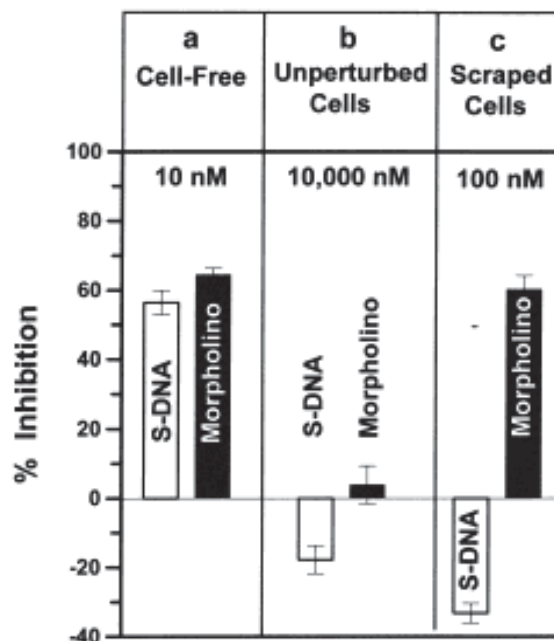


FIG. 6. Cell-free and in-cell activities. (a) Cell-free translations as per Figure 3b with 10 nM oligos. (b) Inhibition of in-cell expression of globin/luciferase mRNA in unperturbed HeLa cells treated for 16 hours with oligos at a concentration of 10,000 nM in the extracellular medium. (c) Inhibition of in-cell expression of globin/luciferase mRNA in HeLa cells scrape-loaded with oligos at a concentration of 100 nM in the extracellular medium.

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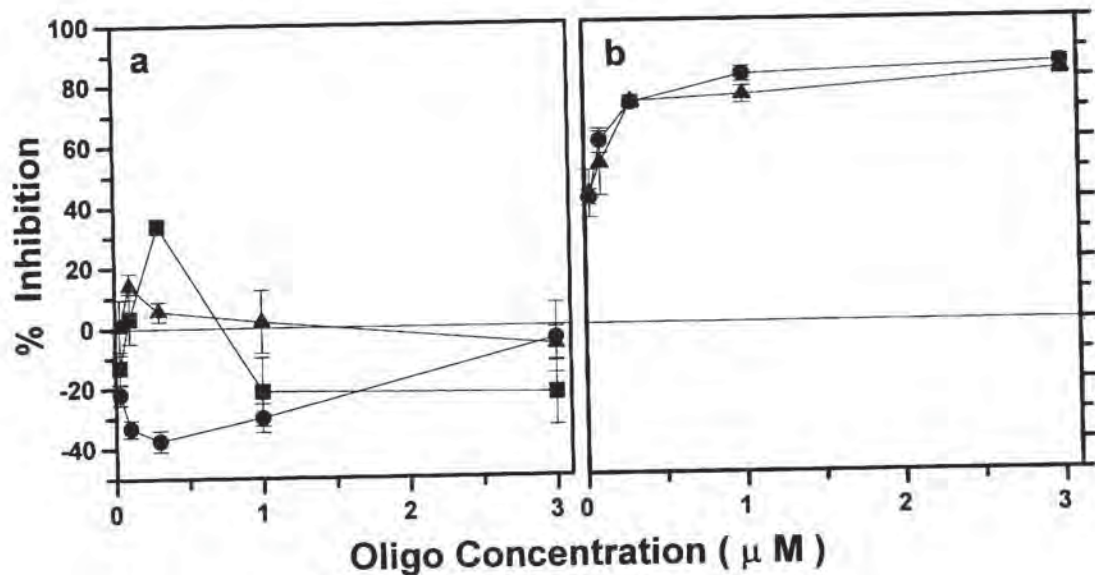


FIG. 7. Efficacies in cells. (a) S-DNA oligos. (b) Morpholino oligos. Closed circles, globin antisense oligo with globin mRNA in scraped cells; closed triangles, HBV antisense oligo with HBV mRNA in scraped cells; closed squares, globin antisense oligo-LIPOFF)CTETm complex with globin mRNA in unperturbed cells.

TECTINTM Figure 7a shows the activities of S-DNA antisense oligos in scraped cells containing the target sequence for the respective oligos. It also shows the activity of an S-DNA antisense oligo complexed with LIPOFECTINTM in unperturbed cells containing the target sequence for that oligo. Figure 7b shows the activities of corresponding Morpholino antisense oligos in scraped cells.

We also used this scrape-load oligo delivery procedure to test for nonantisense activities of S-DNA and

Morpholino oligos in cells. To this end, oligos were delivered into cells containing a luciferase-coding plasmid that lacked the target sequence for said oligos. Specifically, globin-targeted antisense oligos were delivered into cells containing the HBV plasmid, and HBV-targeted antisense oligos were delivered into cells containing the globin plasmid. Figure 8a shows the non-antisense activities of S-DNA oligos in scraped cells, and Figure 8b shows the nonantisense activities of corresponding Morpholino oligos.

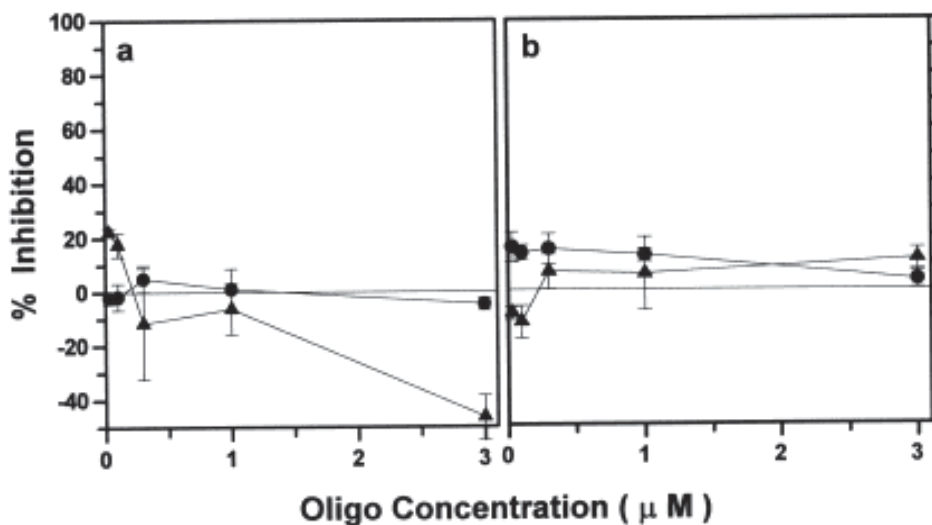


FIG. 8. Non-antisense activities in cells. (a) S-DNA oligos. (b) Morpholino oligos. Closed circles, HBV antisense oligo with globin mRNA in scraped cells; closed triangles, globin antisense oligo with HBV mRNA in scraped cells.

DISCUSSION

Cell-free results

In the cell-free studies in the presence of RNase H, both SDNA and Morpholino antisense oligos achieved reasonable efficacies at low oligo concentrations, averaging about 25% for S-DNAs and 50% for Morpholinos at just 10 nM, with both structural types achieving nearly quantitative inhibition of their targeted mRNAs at 100 nM.

With regard to specificity of S-DNAs, in the most stringent test of specificity (Fig. 4a), the sequence-specific component of the inhibition by the globin-targeted S-DNA dropped below 50% at an oligo concentration of 100 nM and approached 0% at an oligo concentration of 1000 nM. Further, the S-DNAs exhibited significant nonantisense effects at an oligo concentration of 100 nM, and these effects dominated at oligo concentrations above about 500 nM. It is noteworthy that Cazenave et al. (1989) have also reported poor sequence specificity by S-DNAs in a cell-free translation system.

In comparison with the S-DNAs, the Morpholino oligos afforded greater sequence specificity over a far broader concentration range, evidenced by the sequence-specific component of the inhibition by the globin-targeted Morpholino oligos averaging about 90% at an oligo concentration of 3000 nM. Further, the Morpholino oligos exhibited little nonantisense activity over the full 10 nM-3000 nM concentration range.

In-cell results

Although the S-DNA antisense oligos at a concentration of 100 nM afforded near quantitative inhibition of their targets in the cell-free system, in studies with scrape-loaded cells containing the same targets, these S-DNA oligos appear not to inhibit translation of their targeted mRNA at S-DNA concentrations up to 3000 nM in the extracellular medium. At the higher concentration of 10,000 nM (data not shown), however, the globin-targeted S-DNA antisense oligo did afford a modest 34% inhibition. In the presence of LIPOFECTINTM, the S-DNA antisense oligo was moderately inhibitory (33%) at 300 nM but appeared inactive at lower and higher concentrations.

In contrast to the S-DNAs, the Morpholino antisense oligos gave results similar to what they showed in the cell-free system, affording significant inhibition of their targets at all concentrations tested, ranging from 41% at 30 nM to 85% at 3000 nM. It should be noted that in this scrape-load procedure, our fluorescence microscopy observations indicate that oligos enter only about 85% of the cells (Partridge et al., 1996). Thus, at extracellular concentrations of 300 nM and above, target inhibition appears to be nearly complete in those cells that received oligo.

In the specificity study wherein oligos were delivered into cells lacking their targeted sequences, the Morpholino oligos also behaved as one would predict from the cell-free results, exhibiting little inhibition of nontargeted mRNAs over the full 30 nM-3000 nM concentration range. In regard to specificity of Morpholinos in cells, we have reported previously that the globin-targeted four-mismatch control Morpholino oligo (Fig. 2), at concentrations ranging from 30 nM to 3,000 nM in the extracellular medium, does not inhibit its target in scrape-loaded cells (see Figure 7 in Partridge et al., 1996).

It is puzzling that in the cell-free system, both Morpholino and S-DNA antisense oligos achieved near quantitative target inhibition at oligo concentrations of 100 nM, but in the in-cell system, only the Morpholino oligos achieved high efficacy (near quantitative inhibition at 300 nM), with the S-DNAs affording only very low efficacy (less than 50% inhibition at 10,000 nM). Our first guess as to basis for this discrepancy was that our scrape-load procedure might be effective for delivering non-ionic Morpholinos into the cytosol/nuclear compartment of cells but much less effective for delivering polyanionic S-DNAs. However, subsequent fluorescence microscopy studies with fluorescein-tagged Morpholino and S-DNA oligos indicated that in our scrape-load procedure, the S-DNAs entered the cytosol/nuclear compartment even better than did the Morpholinos.

Another possible explanation for the great discrepancy between in-cell efficacies of these two antisense structural types comes from recent evidence reported by scientists at Gilead (Moulds et al., 1995) that suggests the presence of a strand-separating activity in mammalian cells. Specifically, they have shown that when unmodified S-DNAs are preannealed with their target mRNAs and then microinjected into either the nucleus or the cytoplasm of cultured cells, the blocked mRNAs are effectively reactivated, evidenced by translation of their protein products. In this context, although the duplex of the polyanionic S-DNA and its target RNA appears to be readily recognized and acted on by this strand-separating activity, we postulate that the corresponding duplex of the Morpholino oligo and its target RNA may be immune to this strand-separating activity because the structure of the non-ionic Morpholino component of the duplex differs so radically from that of natural nucleic acids.

Other properties of Morpholino oligos

In addition to their cell-free and in-cell efficacy and specificity properties detailed here, it is noteworthy that these nonionic Morpholino oligos are surprisingly water soluble (1 g of representative 22-mer dissolves in 4 ml water), are immune to a wide variety of degradative enzymes and serum (Hudziak et al., 1997), and in large-scale production are expected to be substantially less expensive to produce than S-DNA and other DNAlike antisense structural types (Summerton, 1989, 1992). Because of this composite of desirable properties, Morpholino oligos may be particularly suited for antisense research and therapeutic applications.

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Review

Morpholino antisense oligomers: the case for an RNase H-independent structural type

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Abstract

RNase H-competent phosphorothioates (S-DNAs) have dominated the antisense field in large part because they offer reasonable resistance to nucleases, they afford good efficacy in cell-free test systems, they can be targeted against sites throughout the RNA transcript of a gene, and they are widely available from commercial sources at modest prices. However, these merits are counterbalanced by significant limitations, including: degradation by nucleases, poor in-cell targeting predictability, low sequence specificity, and a variety of non-antisense activities. In cell-free and cultured-cell systems where one wishes to block the translation of a messenger RNA coding for a normal protein, RNase H-independent morpholino antisense oligos provide complete resistance to nucleases, generally good targeting predictability, generally high in-cell efficacy, excellent sequence specificity, and very preliminary results suggest they may exhibit little non-antisense activity. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Antisense; Morpholino; RNase H-independent; Specificity; Efficacy; Delivery

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1. RNase H cleavage: origins of the broad acceptance of S-DNAs

A key requirement for effective antisense oligos is that they remain intact for many hours in the extra-cellular medium and within cells. The methylphosphonate-linked DNA analogs developed by Miller and Ts'o in the late 1970s constituted a major advance in the emerging antisense field by providing the first antisense type having good stability in biological systems [1]. However, concerns subsequently developed that methylphosphonates might be inadequate for many antisense applications, particularly therapeutics, because of their low efficacy – typically requiring concentrations in excess of 20 μ M for good activity in a cell-free translation system [2]. Some time later phosphorothioate-linked DNA analogs (S-DNAs) were introduced [3]. These S-DNAs were enthusiastically embraced because they achieved good efficacy at concentrations a 100-fold lower than corresponding methylphosphonates [4]. The S-DNAs also had good water solubility, reasonable resistance to nucleases, and they were readily prepared on standard DNA synthesizers with only modest modification of the oxidation step.

The surprisingly high efficacy of the S-DNAs relative to methylphosphonates was not readily explained by their moderately higher target binding affinities. Investigations into this large discrepancy in efficacies led to the discovery that while methylphosphonates and most other antisense types act only by a *steric block* mechanism, DNA and S-DNA oligos instead act predominantly by an *RNase H-cleavage* mechanism wherein after the oligo pairs to its RNA target sequence the enzyme RNase H can cleave the paired RNA target sequence [5]. It was also discovered that those structural types which

function only by a steric block mechanism (*RNase H-independent* types) are generally effective in blocking translation only when targeted against mRNA sequences in the region extending from the 5' cap to a few bases past the AUG translational start site (see Fig. 2). In contrast, structural types which utilize an RNase H-cleavage mechanism (*RNase H-competent* types) could also be effective against target sequences elsewhere in the RNA transcript of a gene. Representative RNase H-competent and RNase H-independent antisense types are shown in Fig. 1.

The explanation for these differing targeting properties lies in the mechanism of protein translation. In eukaryotic systems an initiation complex recognizes and binds to the 5' cap structure and then scans down the 5' leader sequence until it encounters the AUG translational start site, at which point the full ribosome is assembled, followed by translation of the amino acid coding region of the mRNA. Antisense oligos apparently can physically block progression of the initiation complex down the mRNA leader and block assembly of the ribosome at the AUG translational start site. However, once ribosome assembly occurs at the translational start site that ribosome is capable of displacing almost any bound antisense oligo it encounters as it traverses the amino acid coding region of the mRNA. Presumably this oligo displacement is effected by the very robust ATP-driven unwindase activity of translating ribosomes [6].

Thus, most RNase H-independent antisense types can block translation only when targeted against sequences in the region from the 5' cap to about 25 bases past the AUG translational start site of an mRNA. In contrast, RNase H-competent oligos can also be effective against sequences elsewhere in the RNA transcript by virtue of their effecting deg-

radation of the paired RNA target sequence by RNase H.

To summarize: Based on the higher efficacy and greater targeting versatility of S-DNAs relative to the early RNase H-independent oligos, many workers in the antisense field have concluded that RNase H competency is essential for good antisense activity. This, combined with their ready availability and low cost, have established S-DNAs as the structural type currently most used in antisense studies.

2. Limitations of S-DNAs

With continued study of S-DNAs it is now widely recognized that their good efficacy and targeting versatility are counterbalanced by a variety of disadvantages.

2.1. Degradation

S-DNA oligos are sensitive to nucleases, being degraded in biological systems over a period of hours [7]. Such instability can complicate interpretation of experimental results and may require either shorter-than-desired experiments or multiple dosing.

2.2. Cleavage of non-targeted RNA sequences

RNase H cleaves DNA/RNA and S-DNA/RNA duplexes as short as 5 or 6 base pairs in length and is highly active against such duplexes only 9–10 base pairs in length [8]. As a consequence, essentially every RNase H-competent oligo has the potential to form transient complexes with and induce cleavage of 'non-targeted' cellular sequences having partial homology to the intended target RNA. It seems reasonable to expect that this RNase H cleavage could compromise the sequence specificity of S-DNAs. In simple cell-free translation systems with added RNase H poor sequence specificity is indeed seen with S-DNAs [9]. This same RNase H cleavage might also be expected to cause disruptions in more complex cellular systems and in patients.

2.3. Promiscuous binding

While both DNA and S-DNA support RNase H

cleavage, because DNA oligos undergo rapid degradation in biological systems S-DNAs have become by default the choice for RNase H-competent antisense oligos. The problem this presents is that the pendent sulfurs in the phosphorothioate linkages of S-DNAs interact with a wide variety of proteins, including laminin, bFGF, protein kinase C, DNA polymerase, telomerase, fibrinogen, phospholipase A₂, HIV gp120, HIV reverse transcriptase, CD4, *Taq* polymerase, T4-polynucleotide kinase, fibronectin, many tyrosine kinases, and proton-vacuolar ATPase [10]. For this and other reasons S-DNAs can cause multiple non-antisense effects.

In addition, S-DNAs containing the sequence Pu-Pu-C-G-Py-Py have been shown to trigger B cell activation [11] and S-DNAs containing four or more contiguous guanines have been shown to form a tetrameric complex which can cause a variety of non-antisense effects [12]. S-DNAs within cells have also been reported to rapidly induce Sp1 transcription factor [13].

The non-antisense effects caused by S-DNAs can result in control oligos exhibiting biological activities on a par with that of the antisense oligos [14]. Further, because S-DNAs can effect multiple non-antisense activities it is difficult to confirm that a given biological response is truly due to an antisense mechanism – leading to considerable uncertainty and possible misinterpretations in antisense experiments utilizing S-DNAs [15].

To summarize: Because of their sensitivity to nucleases, limited sequence specificity, and multiple non-antisense effects it appears that S-DNAs are less than optimal antisense tools.

3. Is RNase H competency necessary?

3.1. Efficacy

A key property of the RNase H-competent S-DNAs which led to their broad adoption by the antisense community was their greatly increased efficacy (likely a consequence of their RNase H competency) relative to methylphosphonates. However, since then at least two RNase H-independent types (PNAs [16] and morpholinos [17] shown in Fig. 1) have been developed which often match or exceed the efficacy

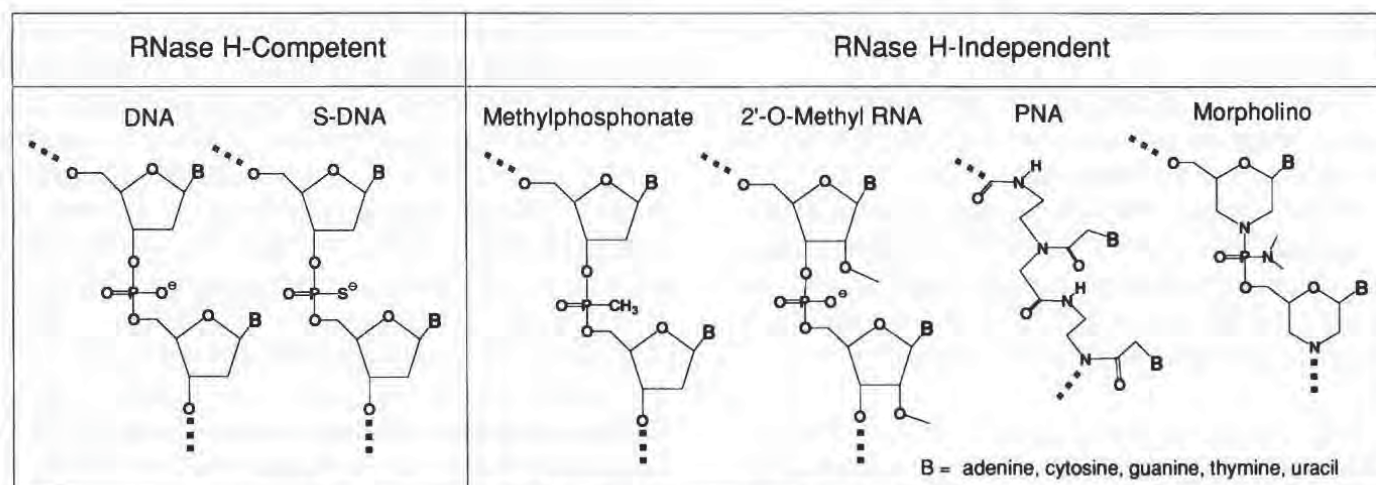


Fig. 1. Representative RNase H-competent and RNase H-independent types.

of S-DNAs in a cell-free translation system when said oligos are targeted to sequences in the region from the 5' cap to about 25 bases 3' to the AUG translational start site [9].

In our cultured cell test system these advanced RNase H-independent antisense types (morpholinos and PNAs) show an even greater efficacy advantage over the RNase H-competent S-DNAs. To illustrate, both S-DNA (RNase H-competent) and morpholino (RNase H-independent) antisense oligos, all of which had been shown to be highly active in a cell-free translation system, were scrape-loaded into HeLa cells and assessed for efficacy in blocking their respective RNA target sequences therein. In this study in cultured cells [18] the two different morpholinos had IC_{50} values of about 60 nM and near quantitative target inhibition at 300 nM. In contrast, neither of the corresponding S-DNAs achieved significant target inhibition within cells at concentrations up to 3000 nM. In similar experiments we have found PNAs (another advanced RNase H-independent type) to exhibit a similar large efficacy advantage over S-DNAs in scrape-loaded cells (unpublished results).

In a different study involving inhibition of TNF- α , Kobzik and coworkers also found morpholinos to achieve appreciably higher efficacies than corresponding S-DNAs in cultured cells [19].

3.2. Targeting versatility

While S-DNAs and other RNase H-competent

antisense oligos can target and destroy (via RNase H cleavage) sites throughout the RNA transcript of a gene, including splice sites, only RNase H-independent antisense oligos, such as morpholinos, can effect correction of splicing errors [20].

Both RNase H-competent and RNase H-independent types can be used to block translation of any specific mRNA by targeting the 5' leader/translational start region of that mRNA.

It is commonly assumed that only S-DNA and other RNase H-competent oligos are suitable for studying point mutations and polymorphisms more than about 20 nucleotides 3' to the translational start site in mRNAs. However, this perceived limitation of RNase H-independent oligos can be circumvented by using a gene switching strategy (P. Morcos, *Methods Enzymol.*, in press). Typically this entails using cells containing a normal gene and transfecting in a plasmid containing a mutant or polymorphic form of that same gene. Key to this scheme is to use a leader sequence in the transfected gene's mRNA which differs by at least a few bases from the leader sequence of the endogenous gene's mRNA. One then uses a high-specificity RNase H-independent antisense oligo, such as a morpholino, to selectively block translation of either the endogenous or the exogenous mRNA, after which one assesses for phenotypic changes. By this means one can exploit the exceptional specificity of the RNase H-independent morpholino antisense oligos to carry out rigorous and well controlled studies of a wide variety of mutations and polymorphisms positioned anywhere in the mRNA.

3.3. Availability

Another factor which led to the widespread use of S-DNAs was their ready availability at moderate prices from commercial sources. While lack of commercial sources, high prices and slow delivery have in the past been significant barriers to use of advanced RNase H-independent antisense types, this situation is now changing. Both PNAs [21] and morpholinos [22] are now commercially available, and prices of morpholinos are now competitive with prices of advanced mixed-backbone S-DNAs (i.e., chimeras).

To summarize: Relative to S-DNAs, properly targeted morpholinos often achieve equal or better efficacy in cell-free systems and often achieve substantially better efficacy in cultured cells; of the two types only morpholinos can be used for correcting splicing errors; a new gene switching strategy gives morpholinos targeting versatility on a par with S-DNAs for selected applications; and morpholinos are now commercially available at moderate prices with reasonable delivery times.

4. Advantages of RNase H-independent morpholino antisense oligos

4.1. Predictable targeting

A problem which has plagued antisense research with S-DNAs is the difficulty of predicting which antisense sequences will be effective in cells. As a consequence, multiple S-DNAs may need to be prepared and empirically tested in order to identify an oligo with good in-cell activity [23,24]. Further, when one does find an effective oligo through such an empirical search it is not unusual to find that it is targeted in a region of the RNA, such as the 3' untranslated region [25], which one would not normally expect to afford inhibition by an antisense mechanism. The poor showing of those many antisense oligos which do not show good efficacy in such searches has been attributed to their having restricted access to their RNA target sequences within cells due to secondary structure of the RNA [26] and/or due to proteins bound to the RNA. Alternatively, this lack of inhibitory activity by many S-DNAs could be due to some activity which disrupts RNA/S-DNA du-

plexes within cells [27] or due to S-DNAs being efficiently sequestered in a partially sequence-specific manner by some nuclear structure [28], or due to activation of Sp1 transcription factor [13] which acts to overshadow the translational inhibition by the S-DNA.

A need to sift through multiple S-DNAs in order to find one that effectively inhibits its target in cultured cells seriously limits the utility of S-DNAs as routine tools for the study of gene function and control. Further, in the absence of rational and reliable targeting rules for S-DNAs and in light of their partially sequence-specific non-antisense effects, the need to test multiple S-DNAs in order to find an effective one raises the specter that one may not be selecting for an accessible antisense target, but instead one may be selecting for an oligo sequence effective to generate some non-antisense activity which is then misinterpreted as the desired antisense effect [15].

In contrast to the difficulty in predicting effective targets for S-DNAs in cultured cells, we have found targeting of morpholinos (lacking undue self-complementarity) to be reasonably predictable both in cell-free systems and within cultured cells. This is illustrated by a targeting study using a target mRNA comprising an 85-base segment of the leader sequence of hepatitis B virus (HBV) fused to the coding sequence of firefly luciferase [18]. This HBV leader sequence presents a substantial targeting challenge because it contains a region of quite stable secondary structure extending from positions -47 to +3 (where +1 is the A of the AUG translational start site). Experimental procedures and the mRNA target used in this study are detailed in [18].

Fig. 2a shows the 5' leader region and 24 bases of the amino acid coding sequence of this mRNA, and indicates with bold lines the target sequences for seven of the morpholino oligos tested in this study. Fig. 2b shows the linear positioning of the morpholino antisense oligos along this HBV-luciferase mRNA, as well as each oligo's percent inhibition achieved in a cell-free translation assay, with oligo present at a concentration of 1 μ M and target mRNA present at 1 nM.

A representative subset of these morpholinos were tested in cultured cells stably transfected with a plasmid coding for the same HBV/luciferase mRNA construct. Fig. 2c shows inhibition of luciferase produc-

tion in cells scrape-loaded in the presence of 3 μ M morpholino oligos, assessed as described in [29].

The results in Fig. 2b show that the tested morpholinos were reasonably effective along the entire 5' leader and up to a few bases 3' to the AUG trans-

lational start site. Oligos targeted to sites beginning more than 20 bases 3' to the AUG translational start site showed no significant activity. Of particular note, the results demonstrate that oligos 3, 4, and 5 appear to have effectively invaded the quite stable secondary structure within the HBV leader sequence.

The results shown in Fig. 2c suggest that those morpholinos which are effective in a cell-free assay are generally also effective in cultured cells, while those morpholinos which are inactive in the cell-free assay (i.e., those targeted more than a few bases 3' to the translational start site) are also inactive in cultured cells.

It should be noted that the IC_{50} of morpholinos in the cell-free translation system are typically about 3–7-fold lower than the IC_{50} in scrape-loaded cells. We postulate that this reflects limited entry of oligos through the very small [30] transient [29] holes believed to be generated in the cell membrane during the scrape-load procedure.

A possible basis for this relatively predictable targeting of morpholinos, even in regions of quite stable secondary structure, may be their high affinity for RNA. Fig. 3 illustrates the comparative affinities of S-DNA, DNA, and morpholino 20-mer oligos for their complementary RNA (assay conditions detailed in [9]).

I postulate that the apparent ability of long morpholino oligos to effectively invade RNA secondary

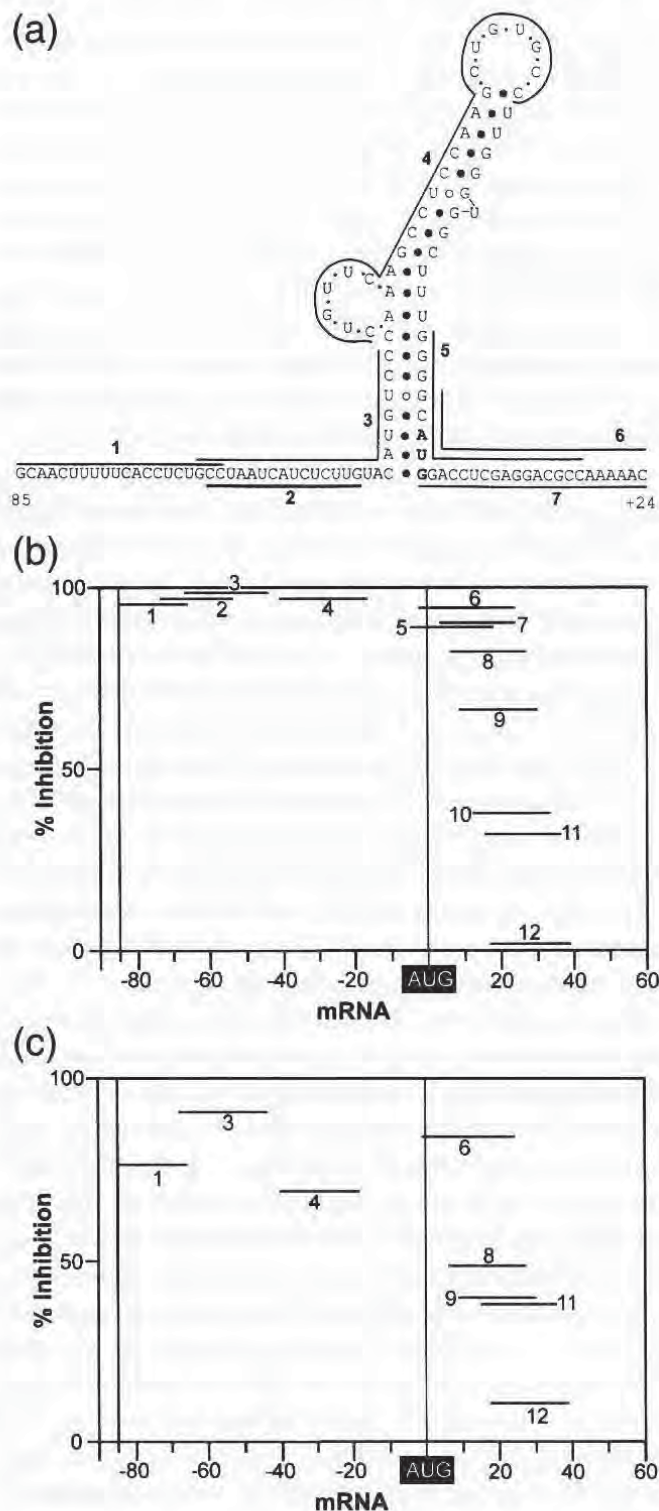


Fig. 2. Translational inhibition as a function of target position on HBV/luciferase mRNA. (a) Leader and translational start region of mRNA with target sequences for oligos 1–7 indicated by numbered bold lines; (b) percent inhibition of luciferase synthesis in cell-free translation system by 1 μ M morpholino oligos 1–12; (c) percent inhibition of luciferase synthesis in cultured HeLa cells expressing HBV/luciferase mRNA and scrape-loaded in the presence of 3 μ M morpholino oligos 1, 3, 4, 6, 8, 9, 11, and 12. The sequence of the HBV/luciferase mRNA from –85 to +24 is: (–85) 5'-GCAAC-UUUUUCACCU-CUGCCUAA-UC-AUCUCUUGUA-CAUGUCCCAC-UGUUCAAGCC-UC-CAAGCUGU-GCCUUGGGUG-GCUUUGGGGC-AUGGAC-CUCG-AGGACGCCAA-AAAC (+24). The 14 oligos used in this experiment are targeted against the following sequences in this mRNA: oligo 1 (–85 to –66); 2 (–73 to –53); 3 (–68 to –44); 4 (–41 to –18); 5 (–5 to +18); 6 (–2 to +24); 7 (+3 to +24); 8 (+6 to +27); 9 (+9 to +30); 10 (+12 to +33); 11 (+15 to +36); 12 (+18 to +39); 13 (+192 to +216); 14 (+528 to +553). Oligos 12, 13, and 14 showed no significant activity in either the cell-free or cultured-cell tests.

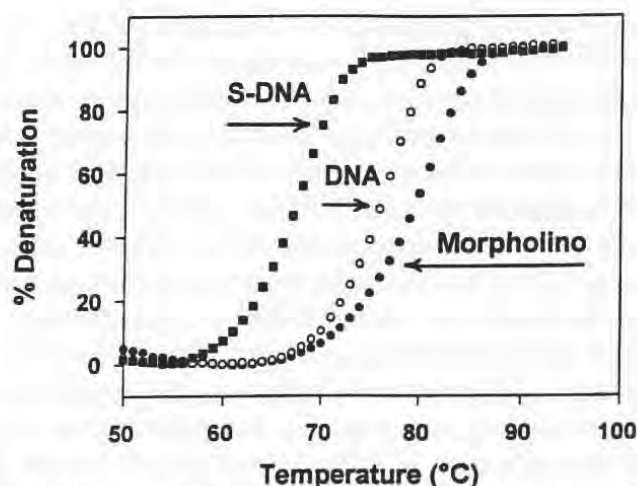


Fig. 3. Thermal transitions of 20-mer oligo/RNA duplexes. Oligo sequence: 5'-GGUGGUCCUUCUCAGUCGG (T replaces U in DNA and S-DNA).

structures may be due at least in part to their high RNA binding affinities – which should favor displacement of short RNA/RNA duplexes to form appreciably longer high-stability RNA/morpholino duplexes. Conversely, S-DNAs, with their substantially lower affinity for RNA, are expected to be much less effective in invading RNA secondary structures.

Use of long morpholinos is also postulated to better assure there will be single-stranded regions in most target sequences to provide for nucleation of pairing.

4.2. Reliable efficacy in cultured cells

As noted above, we have found that morpholino antisense oligos which exhibit good activity in a cell-free translation system also generally exhibit correspondingly good activity when scrape-loaded into cultured animal cells [18]. Similar correspondence between cell-free and in-cell activities of morpholinos has been reported by Kobzik and coworkers at Harvard [19] and by Kole and coworkers at the University of North Carolina (R. Kole, submitted for publication). While I have less experience with PNAs, nonetheless, the few PNAs we have tested also showed good correspondence between activity in a cell-free test system and activity in scrape-loaded cells (unpublished results).

For comparison we have also tested the in-cell efficacies of a number of S-DNAs, which had previously been shown to have excellent efficacies in our

cell-free test system (IC_{50} in the 10–30 nM range). In sharp contrast to the case for morpholinos and PNAs, the scrape-loaded S-DNAs typically show little in-cell efficacy in our test system, and then only at concentrations typically over 3000 nM [18]. In fact, at low to moderate concentrations both antisense and control S-DNAs often strongly increase production of the protein product of the targeted mRNA – possibly via activation of Sp1 transcription factor [13] which could then generate a net increase in the targeted mRNA.

Initially we suspected that perhaps the poor in-cell activity by scrape-loaded S-DNAs might be due to their multiple negative backbone charges preventing good cell entry during the scrape-load procedure. However, when fluorescein-labeled oligos were scrape-loaded into cells it was seen that this procedure achieves delivery of S-DNAs as well as or better than delivery of morpholinos [18].

It seems possible that the poor in-cell efficacy we have seen with S-DNAs and the good in-cell efficacy of morpholinos might be at least in part a consequence of the S-DNAs' sensitivity to nucleases [7] and the morpholinos' complete resistance to nucleases [31].

Another possible explanation for the apparent poor activity of S-DNAs in cells relates to RNase H. In our cell-free translation studies we add *Escherichia coli* RNase H (4 units/ml) because S-DNAs are only poorly active in reticulocyte lysates in the absence of added RNase H. Conceivably, in the HeLa cells, which we typically use for our in-cell studies, mammalian RNase H may be less abundant or less active and so the S-DNAs may afford much less activity than would be expected on the basis of their cell-free activities.

Still another possible reason for lower in-cell efficacies of S-DNAs may be that the S-DNAs' complexes with target sequences are being disrupted by some cellular factor, as postulated by Moulds et al. at Gilead Sciences [27], based on experiments wherein they pre-paired high-affinity anionic antisense oligos with their respective target RNAs and microinjected these duplexes into cells. They found that the anionic oligos were stripped off their target RNAs – evidenced by translation of the protein coded by that RNA. Conceivably such a cellular strand-separating factor might strip off anionic S-DNAs but fail to act

on corresponding non-ionic oligo/RNA duplexes, as would be formed when the non-ionic morpholinos bind their target RNA.

An additional contribution to the good in-cell efficacies of morpholinos and the poor in-cell efficacies we have encountered with S-DNAs in scrape-loaded cells may derive from their respective subcellular localization. Specifically, when low concentrations of fluorescein-labeled S-DNAs are scrape-loaded into cells fluorescence microscopy shows them to be largely sequestered in the nucleus. In contrast, fluorescein-labeled morpholinos scrape-loaded into cells are seen to distribute more evenly throughout the cell – though the concentration in the nucleus usually appears to be severalfold higher than in the cytosol. To appreciate the impact these subcellular distributions of oligo may have on antisense efficacies it should be noted that in the nucleus transcription and processing of a pre-mRNA, and transport of the resulting mature mRNA to the cytosol typically occur within minutes, while translation of the mRNA in the cytosol typically continues for hours to days. As a consequence, S-DNAs largely sequestered in the nucleus may have access to their target RNAs primarily in the brief time between transcription and export to the cytosol, while the more evenly distributed morpholinos should have access to their target RNAs both during the RNAs' brief sojourn in the nucleus and during their far longer residence in the cytosol. It seems quite possible that this difference in target access time might contribute to the high in-cell efficacies of morpholinos and PNAs and the low in-cell efficacies of S-DNAs.

It is noteworthy that in fluorescent microscopy studies of the subcellular distribution of oligos we have observed that addition of mounting medium to the cells can cause significant redistribution of the label relative to that in unperturbed cells – in particular, a transition from a perinuclear punctate pattern to a strongly nuclear pattern. Based on the composition of typical mounting media we suspect this redistribution of fluorescence within the cells may be due to osmotic shock and/or pH stress of the cells. To preclude such artifactual effects we view cells with an inverted fluorescent microscope. This allows the cells to remain bathed in normal growth medium during visualization and photography.

4.3. High specificity

A key factor which lured many scientists (and investors) into the antisense field was the hope that a given antisense oligo could, with near perfect specificity, block its targeted mRNA while exerting essentially no other effects on the cell or patient. It was hoped such high specificity by antisense therapeutics would avoid the severe toxicities characteristic of present small-molecule antiviral and anticancer therapeutics [32]. However, these great expectations largely died for many scientists in the antisense field when: (a) it became widely believed that RNase H competency was essential for good efficacy; and (b) the specificity limitations of S-DNAs became widely appreciated.

Achieving reliably high antisense specificity in a predictable manner would also open the door for use of antisense oligos as dependable tools for studying the function and control of genes and for validating new therapeutic targets – commonly the first step in current strategies for small-molecule drug development.

At present there are two quite different approaches to achieving high antisense specificity – which I refer to as the 'shorter-is-better' and the 'longer-is-better' strategies.

4.3.1. Shorter-is-better strategy

In this strategy relatively short high-affinity oligos, typically of the RNase H-competent type, are used to target point mutations or unusual secondary structures in the selected RNA. When targeting a point mutation there is generally a one base difference between the targeted sequence (typically a mutant) and the non-targeted sequence (typically the wild type). Because there is only one base difference, clearly the shorter the antisense oligo the greater the differential between the antisense oligo's binding affinity for the target and non-target sequences. In order to achieve reasonable efficacies with such short sequences, modifications (such as propyne moieties on the pyrimidines [33]) are introduced to increase the oligo's binding affinity. Efficacy can be increased even further by going to a chimeric oligo wherein the weaker-binding RNase H-competent segment of the chimera is bounded on one or both sides by a higher-affinity stretch of derivatized RNA [34]. While this short/

high-affinity strategy can achieve impressive selectivity between the mutant and wild type sequences, statistical considerations suggest that such high-affinity oligos may inadvertently inactivate other sequences in the pool of cellular RNAs – as described in Section 4.3.2. As a consequence, these short high-affinity oligos may be unsuitable as general tools for most studies of function and control of new genes and for target validation programs. This is because in such cases one's objective is generally to predictably inhibit expression of a selected gene without significantly inhibiting the expression of any other gene in the cell.

An interesting variation of the 'shorter-is-better' strategy has been pursued by scientists at Gilead. They have empirically identified a sequence in an RNA with a particular secondary structure which can be inhibited by very-high-affinity antisense oligos as short as 7 bases in length [35]. While statistical considerations would suggest poor specificity from such oligos, these workers report that these oligos do not inhibit their complementary sequence if that sequence is inserted at a different site in the RNA, presumably because effective inhibition is contingent on both the target sequence and the secondary structure encompassing that target sequence. Potentially this very-short/high-affinity/special-target strategy [36] could lead to relatively low cost antisense therapeutics – especially in light of a recent report that such short oligos can be rendered membrane permeable by addition of a suitable lipophilic moiety [37]. However, it seems unlikely they will soon constitute routine tools for studying gene function and control or for validating therapeutic targets because of the difficulty in routinely identifying suitable targets [26].

4.3.2. Longer-is-better strategy

Aside from their potential therapeutic applications (which is outside the scope of this review), probably the greatest value for antisense oligos would be their use as tools for studying gene function and control and for validating new therapeutic targets in small-molecule drug development programs. Such applications generally call for an antisense oligo which can predictably achieve near quantitative inhibition of its selected target RNA (high efficacy) while avoiding significant inhibition of any other of the host of inherent cellular RNA species (high specificity).

In this regard, conventional wisdom in the anti-

sense field generally holds that one can design for high efficacy, at the cost of reduced specificity, or one can design for high specificity, at the cost of reduced efficacy, but that it is generally not possible to achieve both high efficacy and high specificity.

To the contrary, I contend that by using relatively long oligos of the proper structural type one can achieve both high efficacy and high specificity. To meet this dual efficacy/specificity challenge, I believe the antisense oligo should have a '*minimum inactivating length*' (MIL) sufficient to give a high probability that said oligo will inactivate essentially no inadvertent targets in the cellular RNA pool. Here I define the MIL as the shortest length of oligo of a given structural type which achieves substantial target inhibition at concentrations typically achieved in the cytosol/nuclear compartment of treated cells.

In regard to estimating the MIL value required for high specificity by a selected antisense structural type, one needs to have a reasonable estimate of the number of unique-sequence bases in the cell's RNA pool, as well as the approximate percentage of those bases which could potentially be targeted by that selected antisense structural type.

With respect to S-DNAs, it is difficult to estimate a required MIL value due to uncertainties regarding the fraction of bases in a representative RNA which are targetable. On the one hand, S-DNAs combined with RNase H have the potential to bind and degrade sequences anywhere along the length of an RNA strand. However, it is commonly found that a substantial fraction of S-DNAs fail to inhibit their targeted sequences within cells [23,24]. As a consequence, it is not possible to estimate with any assurance the fraction of bases in the cell's RNA pool which are potentially available for inactivation by S-DNAs within cells. Thus, it is not possible to estimate the MIL required for high specificity of S-DNAs.

While predicting effective targets for S-DNAs within cells can be difficult, this appears not to be the case for morpholinos. Specifically, in both cell-free translation systems and in cultured cells we have found morpholino oligos to be effective against the majority of sequences tested to date, even including sequences with quite stable secondary structures, in the region from the 5' cap to about 25 bases 3' to the AUG translational start site of mRNAs – as detailed

in Section 4.1. It should be noted that we know of two exceptions to this relatively good targeting predictability. Morpholinos with significant self-pairing potential (more than four contiguous intrastrand base pairs) and morpholinos with four or more contiguous guanines typically show poor activity, presumably due to intrastrand pairing and to inter-strand complex formation, respectively.

In view of the relatively predictable targeting for morpholinos it should be possible to estimate a realistic lower limit for the MIL value of morpholinos adequate to assure high sequence specificity. To this end, I estimate that on the order of 3–5% of the genome is transcribed in any given cell type. Thus, in a typical line of cultured cells the RNA pool should comprise on the order of 120 million bases of unique-sequence RNA. From an assessment of a representative sampling of human RNA transcripts I estimate that the regions susceptible to inhibition by morpholinos comprise only about 2–5% of that RNA pool (the other 95–98% being introns and sequences more than about 25 bases 3' to the translational start site). Accordingly, to achieve high sequence specificity a morpholino oligo only needs to distinguish its selected target sequence from no more than about 6 million bases of unique-sequence cell RNA (i.e., 5% of 120 000 000).

Table 1 was constructed based on an estimated RNA pool size of 120 million bases, of which 5% are targetable by morpholinos. Values were calculated by the equation given in the legend to Table

Table 1

Estimated numbers of inadvertent targets in the human RNA pool for an RNase H-independent morpholino 25-mer as a function of MIL

MIL	Targets in pool
7	6960
8	1650
9	390
10	92
11	21
12	5
13	1
14	0

$$\text{Inadvertent targets} = \frac{\text{pool complexity}}{4^{\text{MIL}}} \times (\text{oligo length} - \text{MIL} + 1).$$

Pool complexity: 6 000 000 for RNase H-independent oligo type.

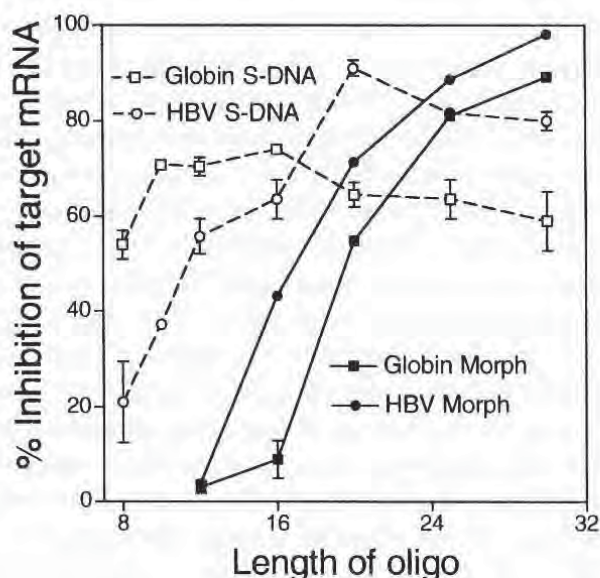


Fig. 4. Activity as a function of oligo length in a cell-free translation system with 300 nM oligo and 1 nM target mRNA. Globin refers to a globin/luciferase mRNA construct and HBV refers to a hepatitis B/luciferase mRNA construct, both detailed in [18]. The HBV target region comprises bases –85 to –56 having the sequence (–85) 5'-GCAACUUUUUCACCUCUGC-CUAAUCAUCUC (–56), and oligos against this region comprise: 8-mer (–85 to –78); 10-mer (–85 to –76); 12-mer (–85 to –74); 16-mer (–85 to –70); 20-mer (–85 to –66); 25-mer (–85 to –61); 30-mer (–85 to –56). The globin target region comprises bases –30 to –1 having the sequence (–30) 5'-CUG-GUCCAGUCCGACUGAGAAGGAACCACC (–1), and oligos against this region comprise: 8-mer (–8 to –1); 10-mer (–10 to –1); 12-mer (–12 to –1); 16-mer (–16 to –1); 20-mer (–20 to –1); 25-mer (–25 to –1); 30-mer (–30 to –1).

1, which factors in the additional sequences inherently present when the oligo length is greater than the MIL value. Table 1 lists the calculated number of inherent cellular RNA sequences expected to be inadvertently inactivated by a morpholino 25-mer antisense oligo having the indicated MIL values.

The values in Table 1 suggest that to achieve very high sequence specificity an RNase H-independent morpholino 25-mer should have an MIL of at least 13 or 14.

Experiments have been carried out to estimate MIL values in a cell-free translation system for both RNase H-competent S-DNAs and RNase H-independent morpholinos. In these activity versus oligo length experiments a set of both S-DNA and morpholino oligos ranging in length from 8 bases to 30 bases were targeted against the same region of

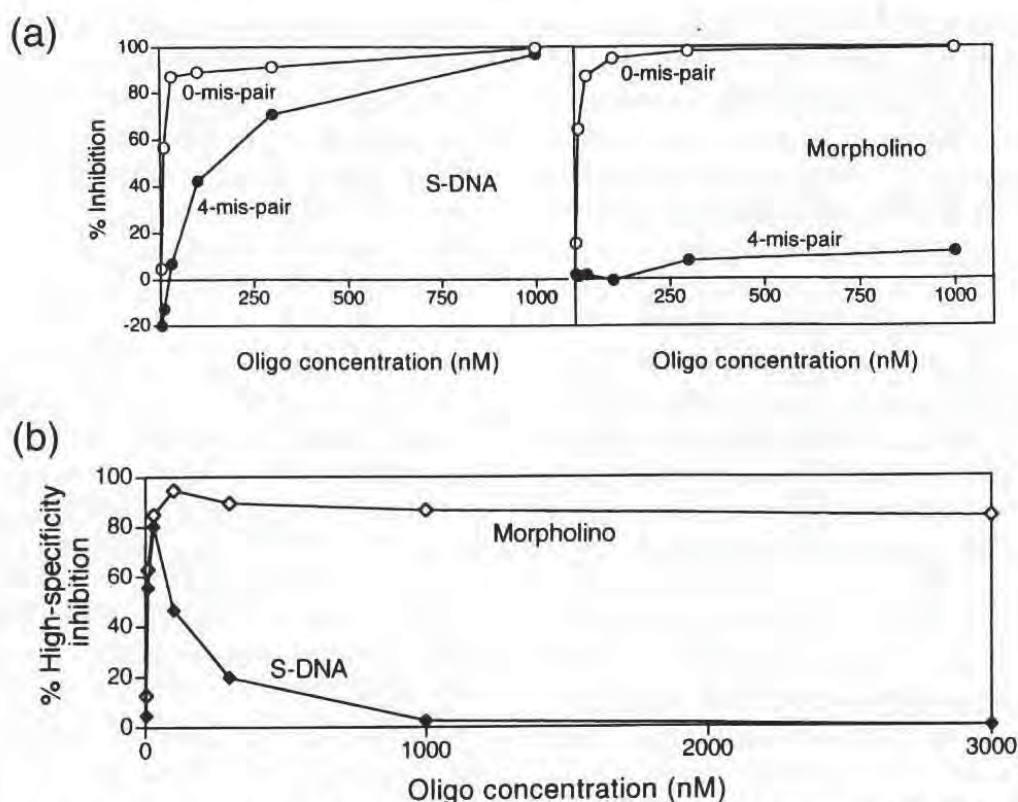


Fig. 5. Sequence-specific inhibition of globin/luciferase mRNA in a cell-free translation system. (a) Inhibition by 0-mispair oligos (open circles) and 4-mispair oligos (closed circles); (b) high specificity component of inhibition = (inhibition by 0-mispair oligo) – (inhibition by 4-mispair oligo); 0-mispair oligo: 5'-GGUGGUUCCUUCUCAGUCGGACUGG; 4-mispair oligo: 5'-GGUCGUUGCUUCUCAGUCCGACAGG.

rabbit α -globin leader sequence, and another set were targeted against the same region of HBV leader sequence. These two sets were assessed in a cell-free translation system for their abilities to inhibit translation of a downstream luciferase coding sequence. The experimental procedures and the RNA targets used in this length versus activity study are detailed in [18]. Fig. 4 shows the respective translational inhibition values at oligo concentrations of 300 nM.

The results in Fig. 4 suggest that under the conditions of this experiment morpholinos against these two target regions appear to have MIL values from about 14 to 17. Since their calculated minimum required MIL value for high sequence specificity in cultured cells is about 13 to 14 for morpholino 25-mers, this bodes well for such oligos achieving excellent sequence specificity. While a morpholino oligo in the 13–14 subunit length range should afford high specificity, if the oligo is only this length it will generally achieve only marginal efficacy. Therefore, to achieve high efficacy one needs to make the oligo

appreciably longer than the MIL. We find that morpholino 25-mers generally achieve good efficacies in the nanomolar to low-micromolar concentration range [38]. Even with these relatively long oligos the values in Table 1 suggest most morpholino 25-mers should have very few or no inadvertent targets in a human cell line.

To test this predicted high specificity of morpholinos we have carried out a highly stringent specificity assay of morpholinos and S-DNAs in a cell-free translation system [18]. In these experiments two oligos of each structural type were used. One oligo of each type was perfectly complementary to its target mRNA (globin leader sequence) to provide a measure of total inhibition achieved by that oligo type. The other oligo of that type incorporated four mispairs to that same target sequence, with the longest run of perfect pairing comprising 10 contiguous base pairs, to provide a reasonable emulation of the estimated level of sequence homology likely to be encountered in the RNA pool within human cells.

Fig. 5a shows the percent inhibition values for these four oligos in the range from 1 nM to 1000 nM.

The difference between the inhibition value for the perfectly paired and the four mispaired oligos at each concentration provides a concentration-dependent measure of the high-specificity inhibition achieved by that structural type. Fig. 5b shows a plot of this high-specificity inhibition component over an extended concentration range (3–3000 nM).

In accord with the predictions from the MIL values in Table 1 and the length versus activity results in Fig. 4, the results in Fig. 5 demonstrate that in this stringent test of specificity the RNase H-independent morpholino 25-mer achieves both high efficacy (IC_{50} less than 10 nM) and high sequence specificity (84–95%) over a broad 3000 nM concentration range.

In contrast, the RNase H-competent S-DNA 25-mer, which the results in Fig. 4 suggests has an MIL value of around 7–9, achieved only modest high-specificity inhibition (47–80%) in a narrow 90 nM concentration range.

To summarize: I postulate that both high specificity and high efficacy can be achieved by an antisense oligo: (a) whose actual MIL is at least as long as the MIL required to achieve high specificity; and (b) whose length is substantially longer than its actual MIL. The results in Fig. 5 suggest that morpholino 25-mers, which satisfy these MIL-related design criteria, do indeed meet the antisense field's long sought dual goals of high efficacy and high specificity over a broad concentration range.

4.4. Little non-antisense activity

As yet we have only limited information concerning possible non-antisense activity of morpholinos – but the very preliminary information we do have suggests morpholinos are free of some, and perhaps all of the non-antisense activities plaguing S-DNAs.

(a) In our in-cell test system low concentrations of S-DNAs, including both antisense and control sequences, often strongly stimulate instead of inhibit production of the protein product from the target mRNAs [18] – possibly due to the documented rapid induction of Sp1 transcription factor by S-DNAs [13]. In contrast, in our in-cell test system corresponding morpholino oligos generally do not significantly stimulate production of the protein product

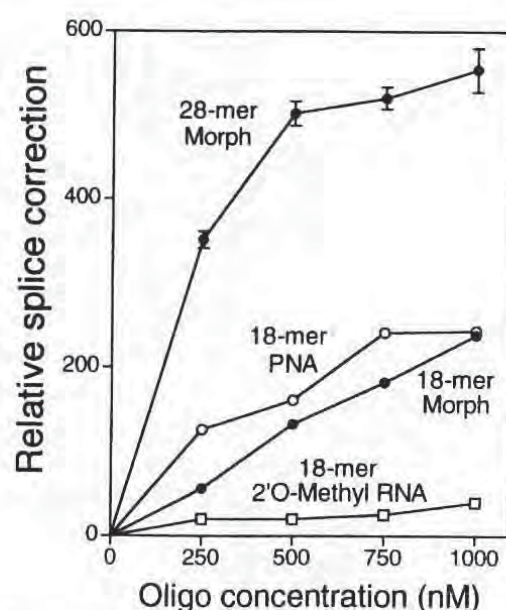


Fig. 6. Luciferase translated from corrected splicing of thalassemic globin/luciferase pre-mRNA in HeLa cells scrape-loaded with antisense oligos. Oligo sequences: 18-mer: 5'-CCTCTTACCTCAGTTACA; 28-mer: 5'-CCTCTTACCTCAGTTACAATTTATATGC.

from the targeted mRNAs [18] and so presumably do not induce Sp1 transcription factor.

(b) S-DNAs containing Pu Pu C G Py Py sequences often mediate B cell activation [40]. In contrast, the same-sequence morpholinos do not activate B cells (A. Krieg, personal communication).

(c) S-DNAs have been reported to have acute LD_{50} values in mice in the range of 100 mg/kg to about 500 mg/kg [39]. In a small ranging study with four mice no acute toxicity was seen following intraperitoneal injection of a morpholino 22-mer at a dose of 800 mg/kg.

5. Positive test system

A long standing limitation in antisense research has been that the available test systems rely on down-regulation. This includes such a crude measure as inhibition of cell growth, as well as assays for inhibition of the synthesis or activity of a particular protein, and assays for degradation of a particular RNA (useful only with RNase H-competent oligos). The difficulty in these negative test systems is that a variety of non-antisense effects can also lead to

down-regulation or what appears to be down-regulation. Even when a reasonable set of control oligos are incorporated into the experiment there can still be significant uncertainty as to whether the observed biological effect is really due to an antisense mechanism. This is because non-antisense effects can also exhibit some degree of sequence specificity – while not being truly specific for the selected target RNA [15,39,41].

The limitations inherent in negative test systems have recently been overcome by the introduction of a positive antisense test strategy by Kole and co-workers [20,42,43]. Kole's 'splice-corrector' strategy entails targeting an RNase H-independent antisense oligo against a mutant site in the pre-mRNA which leads to erroneous splicing. Blockage of that mutant site by the antisense oligo corrects the splicing error, leading to correctly spliced mRNA, which is then translated to give the desired protein. An early version of this test system entailed up-regulation of β -globin and employed readout of both the correctly spliced mRNA and the final β -globin product [42]. A more recent version [43] affords a more convenient luminescence readout from firefly luciferase. Using this new test system, Fig. 6 shows representative results from an experiment measuring relative light units from luciferase synthesized in cultured cells scrape-loaded in the presence of varying concentrations of three different types of RNase H-independent splice-corrector antisense oligos (P. Morcos, *Methods Enzymol.*, in press).

Experiments utilizing this new splice-correction assay should be largely free of the ambiguity which has plagued the antisense field. As such, we find this system to be particularly useful for studies focused on developing effective cytosol/nuclear delivery of antisense oligos. It should be noted that only RNase H-independent oligos can be used in these splice-correction assays because RNase H-competent oligos destroy the mutant pre-mRNA.

6. Delivery

6.1. Delivery into cultured cells

In the 1980s a number of antisense experiments with cultured cells suggested that antisense oligos

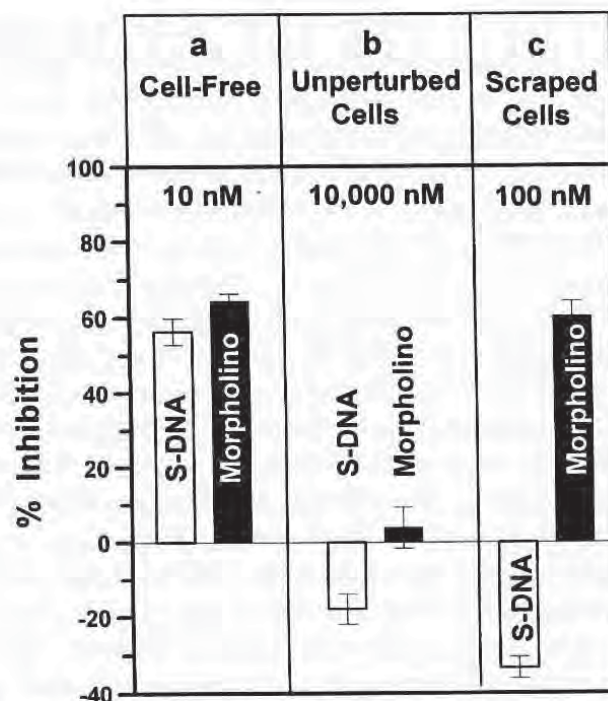


Fig. 7. Translational inhibition by 25-mer antisense oligos targeted against globin/luciferase mRNA: (a) in cell-free translation system with 10 nM oligo and 1 nM mRNA; (b) in unperturbed HeLa cells expressing globin/luciferase mRNA and treated with 10000 nM oligo for 16 h; (c) in HeLa cells expressing globin/luciferase mRNA and scrape-loaded in the presence of 100 nM antisense oligo. Oligo sequence: 5'-GGUGGU-UCCUUCUCAGUCGGACUGG.

readily enter cultured animal cells and have good access to their targeted RNAs therein. These early results led scientists in the antisense field to believe that antisense oligos, and particularly non-ionic types, could readily diffuse across cell membranes. However, by the early 1990s reality reared its ugly head in the form of a number of careful experiments whose results indicated that neither ionic nor non-ionic antisense oligos can diffuse across cell membranes at any reasonable rate [44,45]. Instead, much evidence suggests that antisense oligos enter cultured cells primarily via endocytosis and subsequently most or all of the oligos are degraded, remain trapped in the endosome/lysosome compartment, or are exocytosed from the cell [46].

These sobering findings stimulated broad ranging efforts to develop methods effective for delivering antisense oligos into the cytosol/nuclear compartment of cells. Probably the most used of the resulting delivery methods entails complexing anionic oligos

with liposomes, followed by fusion of these oligo/liposome complexes with cells [47,48]. Another method, which can be used with both ionic and non-ionic antisense types, entails generation of pores in the plasma membrane of cells using streptolysin O under serum-free conditions, and then after oligo has entered the cells, closing those pores by adding serum [49].

A third method which we use extensively entails adding oligos to adherent cells, scraping the cells from the plate, and transferring the scraped cells to another plate where they are allowed to readhere [29]. It is believed that when the cells are scraped from the plate, desmosomes connecting the cells to the plate are pulled out of the cell membranes, leaving very small transient holes in the cytoplasmic membrane [30]. This allows oligos to enter the cytoplasm for about a minute [29] before the holes reseal. This scrape-load procedure is fast, easy, and cheap, it works well in the presence and absence of serum; it causes only minimal damage to cells, and it is suitable for delivering both ionic and non-ionic antisense types. The utility of this scrape-load method in cultured cells is demonstrated in Fig. 7 [18], which shows representative translation inhibition results obtained with S-DNA and morpholino 25-mer antisense oligos in: (a) a cell-free translation system using a low concentration of antisense oligo; (b) in unperturbed cells treated with a very high concentration of antisense oligo; and (c) in cells scrape-loaded with a moderate concentration of antisense oligo.

These results demonstrate that oligos which are very active in a cell-free translation system (IC_{50} of less than 10 nM) fail to show significant activity in unperturbed cells treated with a far higher concentration (10 000 nM) for 16 h. However, the morpholino oligo again shows good activity when cells are scrape-loaded with just one hundredth of that concentration.

The above scrape-load method is restricted to use with adherent cells. Another fast and simple method for introducing antisense oligos into the cytosol of both adherent and non-adherent cells entails pinocytotic loading of cells with a hypertonic solution including sucrose, polyethylene glycol, and the oligo to be delivered into the cytosol of the cell. After a 10 min incubation the hypertonic solution is removed and replaced with a hypotonic solution – which

causes lysis of the pinosomes within the cell [50], thereby releasing the antisense oligo into the cytosol of the cell. A kit for such pinocytotic loading of cells was recently introduced by Molecular Probes (Eugene, OR).

6.2. *Delivery in vivo: the final challenge?*

While several effective methods are now available for delivering antisense oligos into cultured cells, none of the above methods are suitable for delivering antisense oligos into cells *in vivo*. However, contrary to expectations from cell culture work there are reports in the literature that S-DNA antisense oligos injected into animals efficiently gain access to their targeted RNAs within cells – even though the same S-DNAs are unable to achieve the corresponding access in cells in culture. Probably the best documented reports of *in vivo* antisense activity by S-DNAs are those of Monia and coworkers [51,52].

In spite of such reports that antisense oligos can readily enter the appropriate compartments of cells *in vivo*, because of the difficulty in confirming a true antisense mechanism with S-DNAs [15,39,41] and the uncertainties inherent in work with whole animals, I believe it is prudent to continue efforts to develop methods for delivering antisense oligos into the cytosol of cells by methods likely to be suitable for application *in vivo*. Below are two strategies which appear particularly promising for delivery of antisense oligos into the proper subcellular compartment of cells *in vivo*.

6.2.1. *Amphiphilic peptides*

Alain Prochiantz and coworkers in France discovered that a fruit fly transcription factor protein is excreted by cells and efficiently reenters adjacent cells, apparently by directly crossing the plasma membrane into the cytosol, after which that protein migrates to the nucleus. They subsequently discovered that an amphiphilic 16 amino acid sequence in that protein is largely responsible for this transmembrane transport activity [53]. Several other groups have also identified proteins which are excreted and then appear to pass directly across the plasma membrane into recipient cells – including VP22 from herpes simplex virus [54] and galaparan [55].

Pooga and coworkers have recently used two such

Endosome to Cytosol Transport

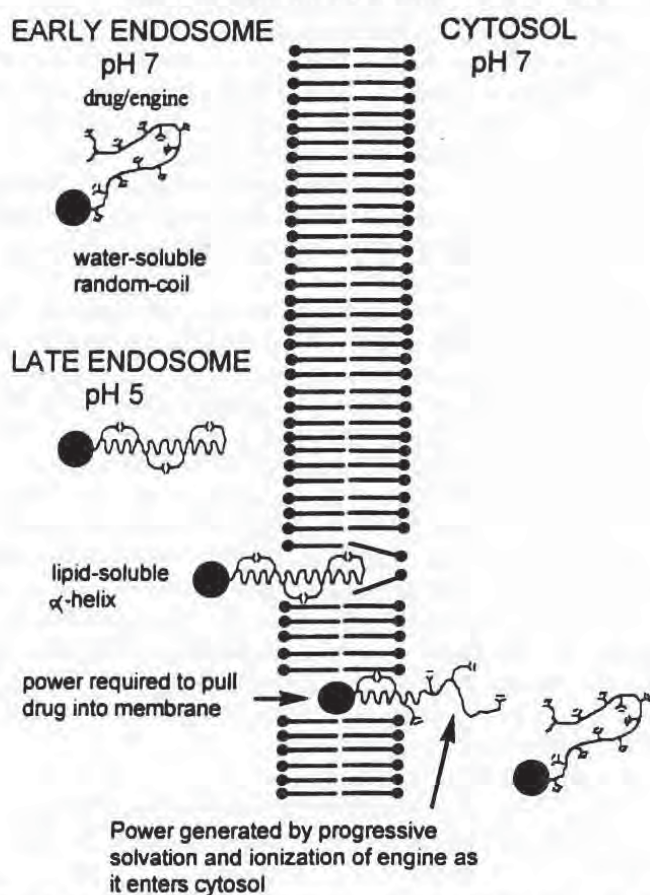


Fig. 8. Mechanism of drug transport from endosome to cytosol by molecular transport engine.

transporter peptides for delivering PNA antisense oligos into biological systems [56]. Specifically, they linked a PNA oligo to an amphiphilic transporter peptide and added this construct to cultured cells. They report that two such constructs utilizing two different amphiphilic transporter peptides efficiently entered cultured cells and achieved an antisense effect therein. They subsequently injected these constructs into rats. Based on an observed biological response in the rats, they infer that these antisense-peptide constructs successfully achieved entry into the proper subcellular compartment and exhibited an antisense effect therein. While these quite recent *in vivo* results have not yet been rigorously confirmed, they nonetheless appear promising because they were carried out with an antisense type (PNA) which appears not to elicit the numerous non-antisense effects commonly seen with S-DNAs.

6.2.2. Molecular transport engine

In a rather different delivery approach I and Dwight Weller have designed from first principles a class of molecular engines for transporting drugs from endosomes to the cytosol of cells [57,58]. The power source for these engines is the pH differential between the late endosome (pH about 5.5) and the cytosol (pH about 7). To convert this pH differential into useful power for drug transport the engines are designed to undergo reversible pH-mediated transitions between a water-soluble form and a lipid-soluble form. The engines contain carboxylic acid groups positioned along a peptide backbone, plus interspersed lipophilic moieties required to fine tune the transition pH and optimize lipophilicity under acidic conditions. A key requirement for achieving good lipophilicity under low-pH conditions is that neighboring carboxylic moieties be properly positioned so as to form doubly H-bonded dimers at low pH – thereby largely masking their polar character.

Fig. 8 illustrates the mechanism by which these molecular transport engines effect passage from the endosome to the cytosol. First, the drug-engine construct in its ionic water-soluble form is endocytosed, after which proton pumps embedded in the endosomal membrane acidify the endosome. When the pH is reduced sufficiently the engine converts from its high-pH ionic water-soluble form to its low-pH non-ionic lipid-soluble α -helical form, which partitions from the aqueous endosomal compartment into the lipid bilayer of the endosomal membrane. Because the engine in its low-pH α -helical form is longer than the membrane is thick (about 36 Å), continuing entry of the engine into the membrane results in the distal end of the engine contacting the pH 7 cytosol, whereupon the engine is actively drawn into the cytosol as it converts back to its high-pH water-soluble form. During its entry into the cytosol the motive force for pulling the attached drug into and through the endosomal membrane is generated by solvation and ionization of the engine at the membrane/cytosol interface.

To date a variety of studies, including solubility, octanol/water partitioning, circular dichroism, transport between low-pH and high-pH compartments separated by a lipid bilayer, and transport directly across the plasma membrane of cells briefly suspended in pH 5.5 medium, indicate that such molec-

ular engines function in the desired manner (Moulton et al., in preparation). Preliminary results with cultured cells also suggest that such an engine may transport a morpholino oligo into the cytosol of cells [57], but these studies (still in progress) are not yet definitive.

It is envisioned that in vivo these oligo-engine constructs will enter cells of the body by the normal endocytotic route, after which the engine will transport the oligo from the endosomes to the cytosol/nuclear compartment. While engines constructed from D-amino acids appear to persist within cells for long periods of time, our preliminary results suggest that engines constructed from L-amino acids are rapidly degraded in the cytosol, presumably by proteosomes to generate innocuous natural amino acids. Thus, it is hoped such engines will carry out their transport function and then be rapidly disposed of in a manner which generates only innocuous natural products.

To summarize: Morpholino antisense oligos appear to meet the requirements for use as effective and predictable tools for studying gene function and control in cultured cells and for validating targets in drug development programs. If and when effective delivery is achieved in vivo, oligos of this type may hold promise as effective, specific, and broadly applicable antisense therapeutics.

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EXHIBIT 27



Morpholinos and PNAs compared

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Introduction

This chapter will compare and contrast the properties and applications of two leading antisense molecules, Peptide Nucleic Acids (PNAs)^{1,2} and Morpholinos^{3,4}. Where appropriate, I discuss the compelling advantages which these two advanced ‘blocker’ types provide relative to ‘modifier’ types of antisense molecules. Some of the properties which are compared include chemical synthesis strategies, chemical stabilities, backbone flexibilities, aqueous solubilities, target selection criteria, target binding affinities, and sequence specificities.

Since the mid-1980s, phosphorothioate-linked DNA oligos (S-DNAs) have dominated the antisense field. For many antisense applications, however, advanced non-ionic oligos provide a far better combination of properties, including stability in biological systems, high efficacy and specificity, lack of toxicity, and freedom from non-antisense effects. Prior to the discovery of PNAs by Nielsen *et al.*, I was the first person to conceptualize and to synthesize morpholinos, recognizing their advantages for antisense chemistry. Morpholinos and PNAs share a number of key properties, such as non-ionic backbones whose structures differ radically from that of nucleic acids, resistance to enzymatic degradation, and high (Morpholinos) or very high (PNAs) affinity for complementary RNA sequences. In the context of diagnostics, a particularly valuable property of both structural types is that they strongly pair to complementary genetic sequences under conditions which disrupt secondary structures of nucleic acids. Another particular advantage of morpholinos, and part of the original impetus to develop them, is the fact that they are relatively cheap to produce; the subunits of Morpholinos can be assembled into antisense oligos via simple and efficient coupling to the morpholine nitrogen, without the expensive

catalysts and post-coupling oxidation steps required in the production of most nucleic acid analogs.

In spite of their many similarities, Morpholinos and PNAs also exhibit significant differences which translate to differing advantages in particular applications. Two key differences which bear on their preferred applications are: 1) PNAs have higher affinities for RNA than do Morpholinos, though both structural types form duplexes with RNA which are more stable than DNA/RNA duplexes, and much more stable than S-DNA/RNA duplexes; 2) Morpholinos are highly soluble in aqueous solutions, generally 5 to 30 mM for 25-mers, depending on sequence, whereas PNAs are typically several hundred-fold less soluble. As a consequence of these differing properties, it appears that PNAs are better suited for high-affinity applications such as targeting short sequences (e.g., the exposed segment of telomerase RNA) and for discriminating between single base differences, as in SNPs (single nucleotide polymorphisms). Conversely, Morpholinos excel in applications which require high aqueous solubility and exquisite discrimination between a targeted mRNA and tens of thousands of non-target mRNAs, such as *in vivo* applications with developing embryos and other complex systems.

Classification of antisense structural types

Sequence-specific nucleic acid-binding oligomers (‘oligos’) can be divided into two categories:

- 1) ‘blocker’ oligos which hydrogen bond to, but do not modify their complementary (targeted) genetic sequences;
- 2) ‘modifier’ oligos which H-bond to and then modify their targeted sequences, either directly by crosslinking or cleavage, or indirectly via RNase H-mediated degradation.

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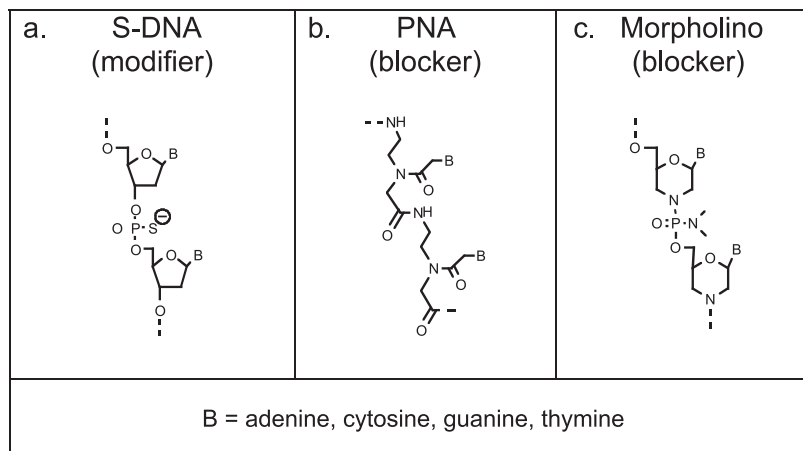


Figure 1. Representative oligo structural types.

Oligos in these two categories differ fundamentally in regard to which sequences they can target in an RNA transcript. Specifically, modifier oligos, exemplified by S-DNAs (Figure 1a), have the potential to target sites anywhere in an RNA transcript. In contrast, blocker oligos, exemplified by PNAs (Figure 1b) and Morpholinos (Figure 1c), are generally ineffective when targeted against intron sequences in a pre-mRNA, unless the target site is immediately adjacent to a splice site. Blocker oligos are also generally ineffective when targeted against amino acid-coding sequences more than about 40 bases 3' to the AUG translational start site in an mRNA. This is probably because once a ribosome completes its assembly at the AUG translational start site, its ATP-dependent unwinding activity becomes capable of displacing nearly all blocker oligos from their targeted sites in the downstream amino acid-coding region.^{1,4,6}

Figure 2a illustrates the limited regions of an RNA transcript which are potentially available for targeting by blocker oligos, currently estimated at about 5% of the total length of the average RNA transcript. Figure 2b illustrates the extensive region of an RNA transcript which is potentially available for targeting by modifier oligos of the types which effect cleavage or degradation of their targeted sequences, essentially 100% of RNA transcripts.

From a targeting perspective, at first glance the modifier oligos (e.g., S-DNAs) appear attractive because they offer the possibility of targeting sites throughout an RNA transcript (Figure 2b). However, in practice this apparent advantage is greatly lessened because selecting effective targets for modifier oligos

is often highly unpredictable and requires much empirical experimentation,^{7,8} presumably because most possible target sites are unavailable due to secondary structures in the RNA transcripts and/or due to other poorly understood factors.

In contrast to the case for modifier oligos, advanced types of blocker oligos (PNAs and Morpholinos) targeted against sequences within their targetable regions (Figure 2a) generally give predictable and effective results.⁹ This is probably because the high RNA-binding affinities of advanced blocker types allow them to efficiently invade the extensive RNA secondary structures common to natural RNAs.⁴

Preparation of morpholinos and PNAs

Subunit synthesis

In the context of custom oligos for research applications, the costs of starting materials, ease and yield of key steps in subunit syntheses, and the costs of oligo assembly and processing are of lesser interest to both producers and users because labor expenses generally dominate the cost of custom oligos. However, for larger-scale applications, such as clinical diagnostics and therapeutics, these factors play a major role in the cost of the finished oligo. Because production costs will be an important factor in clinical applications, these costs will be discussed below.

PNA subunits are prepared from the standard purine (A and G) and pyrimidine (C and T) nucleobases. The key step in producing PNA subunits is selective alkylation on the nitrogen at the 9 position of the purines

and the 1 position of the pyrimidines, as illustrated in Figure 3a. Any lack of selectivity in this alkylation reaction will require careful purification of the desired product. In contrast to alkylation of the bases, adding the N-protected aminoethyl glycine backbone moiety and adding appropriate nucleobase protective groups should be relatively straightforward and should give good yields and purities.

Morpholino subunits are prepared from the natural rA, rC, and rG ribonucleosides. For the fourth subunit, we prefer to use synthetic rT instead of the natural rU because of the positive impact T bases have on RNA-binding affinities of the resulting oligos. The key steps in synthesis of Morpholino subunits are as follows: oxidative opening of the 5-membered ribose ring; closing the resulting dialdehyde on ammonia to give a 6-membered morpholine ring; and reductive removal of the original 2' and 3' hydroxyls, as illustrated in Figure 3b. These three steps are carried out sequentially in a single pot without intervening purifications. Once the ribose-to-morpholine conversion is accomplished, adding appropriate protective groups and adding the chlorophosphoramidate moiety to the original 5' oxygen are relatively straightforward and give good yields and purities. It is noteworthy that in the dry state the final protected/activated Morpholino subunits are stable for many months at -20°C .

Table 1 shows approximate relative costs of the key subunit starting materials for PNA and Morpholino subunits from representative chemical supply companies, and for comparison, subunits for DNA analogs such as S-DNAs.

Both PNAs and Morpholinos enjoy a significant advantage over DNA analogs in regard to cost of starting materials, although some of this cost advantage is lessened due to the costs and yield losses in converting the nucleobases and ribonucleosides to their respective PNA and Morpholino subunit structures.

Oligo assembly

In regard to assembling subunits into oligos, PNAs are typically assembled using a 3-reaction cycle: coupling, capping, and deprotection, as shown in Figure 4a. Including the intervening washes, the subunit addition cycle typically consists of 7 to 9 individual steps.^{10,11,12}

For assembly of Morpholinos, we use a very simple 2-reaction subunit addition cycle analogous to that used for active-ester peptide synthesis. The two reactions are coupling and deprotection, as shown in

Figure 4b. A capping step is not used because coupling and deprotection efficiencies are very high (estimated at about 99.7% for each reaction). Our newest subunit addition cycle for Morpholino oligos comprises these two reaction steps plus three wash steps, for a frugal 5-step assembly cycle carried out on a simple custom-made synthesizer. Through careful selection of solvent combinations, about 80% of the expensive solvent components can be recovered and, after distillation, reused. This recycling allows for cost saving on both the purchase of more solvents and disposal of used solvents. In principle, because of the good stability of the activated Morpholino subunits, much of the excess subunit used to drive the coupling to completion can also be recovered and reused. However, this is only practical in large-scale synthesis of an individual oligo. The recovery and reuse of both subunits and solvents has the potential to substantially reduce oligo costs in large-scale production of Morpholino therapeutics.

Properties of morpholinos and PNAs

Chemical stability

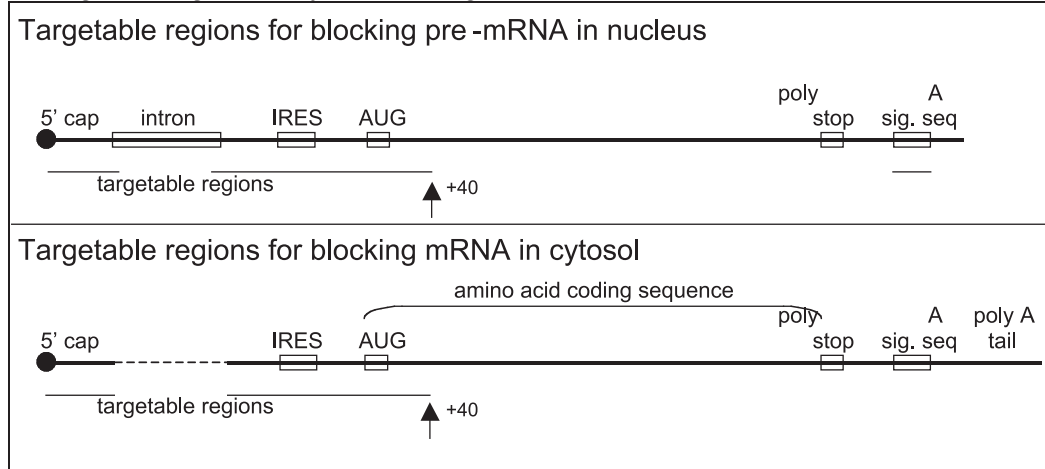
The PNA backbone is stable to strong bases (which would degrade RNA) and to strong acids (which would depurinate DNA). The only significant instability of a PNA chain is when it has a free aminoethyl N-terminus, which occurs briefly during each coupling cycle in oligo assembly¹⁰ and would occur if one failed to cap the N-terminus on completion of the oligo. Because of favorable geometry, this aminoethyl moiety ($\text{pK}_a \sim 10.5$) can cause rearrangements and subunit deletions.¹⁰ Capping or otherwise modifying the N-terminus after completion of oligo assembly effectively stabilizes the finished oligo.

A particular advantage arising from the exceptionally high chemical stability of the PNA backbone is that while the oligo is still on the synthesis resin, a series of amino acids can be added to the oligo to generate a peptide component suitable for enhancing delivery into cells, or suitable for diagnostic applications. The PNA component easily survives the rather harsh conditions required for removal of protective groups on the side chains of that added peptide.

The Morpholino backbone is also stable to strong bases, but in contrast to the acid stability of PNAs, the Morpholino backbone is cleaved by strong acids, such as trifluoroacetic acid. While the sensitivity of Morpholinos to strong acids does impose some limitations

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a. Regions targetable by blocker oligos



b. Regions targetable by modifier oligos

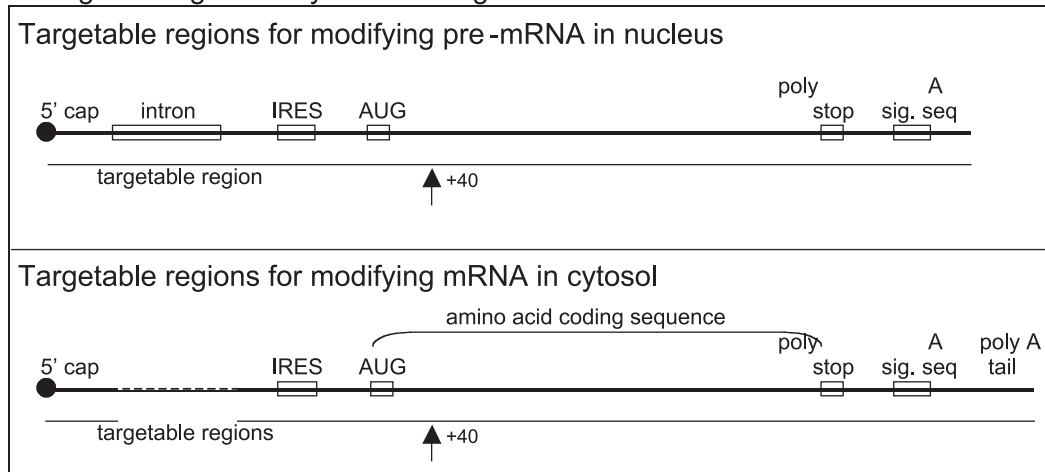


Figure 2. Targetable regions in RNA transcripts.

Table 1. Relative costs of starting materials

Oligo type	Starting material	Approximate relative cost per mole
PNA	Nucleobases	1
Morpholino	Ribonucleosides	2
DNA analogs	Deoxyribonucleosides	70

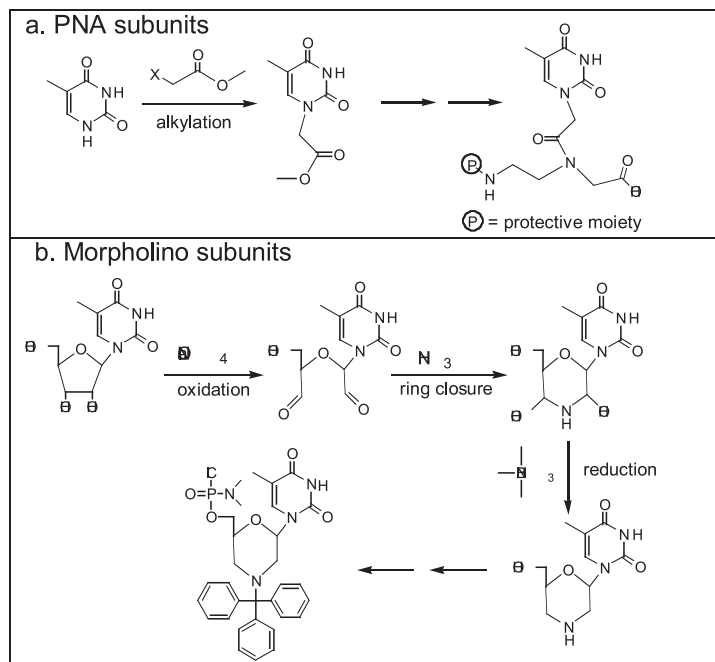


Figure 3. Key steps in subunit synthesis.

on possible chemical manipulations, that same sensitivity also affords at least one significant advantage – a fast and easy way to confirm sequence. In this method, a small portion of synthesis resin with completed oligo still attached is treated with neat TFA (40 minutes at room temperature) to generate on average of one cleavage per oligo chain. The resin is then washed free of TFA and 3'-terminal oligo fragments, and added to concentrated ammonium hydroxide to cleave the resin-bound 5'-terminal oligo fragments from the resin and to deprotect the nucleobases. A mass spectrum directly provides that oligo's sequence simply by tabulating the mass differentials between consecutive fragment peaks.

Biological stability

A major challenge in using antisense oligos has been retention of the integrity of the oligo for a sufficient length of time to achieve the desired experimental or therapeutic effect. For example, bare RNA and DNA oligos introduced into cells undergo substantial enzymatic degradation in a matter of minutes. Various modifications of DNA and RNA backbones (e.g., replacing a sp. pendant oxygen on the phosphorous with a sulfur, as in S-DNAs, or adding an alkyl group to

the 2' oxygen of RNA, as in 2'-O-Methyl RNA) as well as modifying the termini of the oligos to block exonucleases³³ all serve to improve stabilities in biological systems. Nonetheless, for close analogs of DNA and RNA having anionic intersubunit linkages, enzymatic degradation generally remains a significant problem in longer-term experiments.

Unlike the biological instabilities of close analogs of RNA and DNA, the radical design departure from natural nucleic acid structure embodied in PNAs and Morpholinos, coupled with their non-ionic intersubunit linkages, renders them highly resistant to enzymatic degradation in blood and within cells.^{14,15} This stability to enzymatic degradation provides a compelling advantage in applications requiring long-term activity in biological systems, such as in studies in embryos¹⁶ and in therapeutics.

Backbone flexibility

A significant difference between PNAs and Morpholinos is that PNAs have significantly greater backbone flexibility. Specifically, as illustrated by the acyclic conformation on the left side of Figure 5a, PNAs have 7 bonds per subunit which can undergo relatively free rotations, though this may be transiently reduced to

5 freely rotatable bonds for some fraction of the subunits due to intramolecular H-bonding, as illustrated by the pseudo-ring conformation on the right side of Figure 5a. Molecular modeling suggests that the more flexible backbone of PNAs should favor applications such as triplex formation, including PNA/DNA/DNA and PNA/RNA/PNA triplexes. Indeed, PNAs are well known for excelling in such triplex applications.^{17,18}

In contrast to the relatively flexible backbone of PNAs, Morpholinos have a more rigid backbone because they have only 4 bonds per subunit which can undergo relatively free rotations, as illustrated in Figure 5b.

We have found that a key requirement for achieving good antisense activity with high-affinity non-ionic oligos is that they must not contain undue self-pairing potential. Self-pairing is a particular problem for PNAs and to a lesser extent, Morpholinos, because in contrast to the case for oligos having ionic backbones, with non-ionic oligos there is no electrostatic repulsion between the backbones of paired segments to help counterbalance the Watson/Crick pairing. Consequently, when significant self-pairing potential is present in such an oligo, self-pairing is likely to dominate to the exclusion of the desired oligo/target pairing.

In the context of relative backbone rigidities, a likely consequence of the greater flexibility of the PNA backbone, compared to the more rigid Morpholino backbone, is that the PNA oligo can more readily adopt a conformation suitable for self-pairing. To put this in semi-quantitative terms, from our experience in targeting many antisense oligos, it appears that the more flexible PNAs can have no more than about 8 contiguous Watson/Crick H-bonds of self-pairing before they start to suffer a reduction in antisense efficacy. In contrast, the more rigid Morpholinos can have up to about 11 contiguous Watson/Crick H-bonds of self-pairing without suffering significant loss of antisense activity. It should be noted that these numbers of allowable H-bonds are estimates, but may vary up or down by about one H-bond for particular sequences. Table 2 illustrates representative sequences having the approximate maximum amounts of self-pairing which appear to still allow good antisense activity for each of the structural types. Note that an A/T or A/U pair contributes 2 Watson/Crick H-bonds, while a G/C pair contributes 3 Watson/Crick H-bonds.

This modest difference in allowable amount of self-pairing potential for the two structural types results in considerable latitude in selecting effective tar-

Table 2. Approximate maximum allowable amounts of self-pairing

PNA:	-GCA-	-ATTA-
	-CGT-	-TAAT-
Morpholino:	-GCAC-	-ATTAC-
	-CGTG-	-TAATG-

gets for Morpholino oligos¹⁹ as compared to rather severe restrictions imposed in selecting effective targets for PNAs.²⁰ The greater latitude in picking Morpholino targets is particularly advantageous when one wishes to target a relatively long RNA sequence (20 to 30 bases) in order to maximize the chance of complementing a suitable single-stranded region of the RNA, which appears to be needed for efficient initiation of oligo/target pairing.

Aqueous solubility

Conventional wisdom has long held that oligos having non-ionic backbone structures invariably exhibit poor aqueous solubility. Indeed, until recently there was much support for this view, evidenced by the poor aqueous solubilities of the multiple non-ionic structural types shown in Figure 6. Solubilities of these structural types typically are limited to only about 10 to 100 μ M, depending on length and sequence.^{21,22,23,24,25}

While a variety of solubilizing moieties have been added to these oligos to improve their limited aqueous solubilities (e.g., terminal phosphate on Methylphosphonates, polyethylene glycol on DNA carbamates and Morpholino carbamates, one or more lysines on PNAs), nonetheless, the inherent low solubilities of the core oligos often leads to aggregation and precipitation in many biological applications.

Surprisingly, non-ionic Morpholino oligos with freely rotatable bonds in the intersubunit linkage have been found to have excellent water solubility. To illustrate the importance of freely rotatable bonds, at 37 °C carbamates are known to exhibit restricted rotation, while sulfamides and phosphoroamidates are known to exhibit relatively free rotation. In this context, we have found that a Morpholino oligo having the more rigid carbamate intersubunit linkages (Figure 7a) is several hundred fold less water soluble than a corresponding Morpholino oligo containing the more flexible sulfamide or phosphoroamidate intersubunit linkages (Figures 7b–c).

The likely reason for this great difference in water solubility between these Morpholino subtypes is that the restricted rotation of the carbamate linkage largely prevents stacking of the bases,²⁶ so that dissolution in an aqueous environment then requires an energetically unfavorable insertion of the hydrophobic faces of the unstacked bases into water. Conversely, molecular modeling suggests that the free rotations of the phosphorodiamidate and sulfamide linkages should allow excellent stacking of the bases, and this has been confirmed experimentally for the phosphorodiamidate linkage.²⁶ This base stacking translates into excellent water solubility, presumably because the stacking effectively hides the hydrophobic faces of the bases from the aqueous environment.

One practical consequence of the excellent aqueous solubility of phosphorodiamidate-linked Morpholinos (typically 5 to 30 milliMolar for 25-mers) is that a minimal volume of a highly concentrated oligo solution can be injected into quite small eggs or early-stage embryos (e.g., zebrafish), as is required for developmental studies. This high water solubility, combined with exquisite sequence specificity, negligible toxicity, lack of non-antisense effects, and stability in biological systems, have made Morpholino oligos the preferred tools for selective gene knockdown studies in developmental biology.^{9,27}

Salt dependence of binding

An important consequence of an oligo having a non-ionic backbone, as is the case for PNAs and Morpholinos, is that their binding affinity for complementary genetic sequences is relatively insensitive to the ionic strength of the medium. Figure 8a compares T_m (melting temperature) values for 20-mer DNA/DNA and corresponding Morpholino/DNA duplexes as a function of salt concentration. Figure 8b compares T_m values for 20-mer DNA/RNA and corresponding Morpholino/RNA duplexes as a function of salt concentration.

PNA/DNA and PNA/RNA duplexes also exhibit similar independence between T_m and salt concentration.¹

As will be described in the section below on 'applications', this independence between T_m and salt concentration provides a dramatic advantage over classical anionic DNA and RNA oligomers and polymers in probe diagnostic applications.

RNA-binding affinity

Blocker-type oligos must tightly bind to their targeted RNA sequences in order to prevent RNA processing (e.g., splicing), readout (translation), or other functions (e.g., extension of telomers) of their targeted RNA transcripts. Figure 9 shows thermal transitions of various oligo/RNA duplexes (20-mers) at physiological salt concentration.

For comparison, we have found that at physiological salt concentration, PNA/RNA duplexes typically have thermal transitions similar to that of 2'-O-Methyl RNA/RNA duplexes.

From Figure 9, one can see that Morpholino/RNA duplexes are more stable than corresponding DNA/RNA duplexes, and much more stable than corresponding S-DNA/RNA duplexes. While not included in Figure 9, under the same conditions we have found that PNA/RNA duplexes typically have substantially greater stability (about 8° to 10°C for 20-mers) than the already high stability of Morpholino/RNA duplexes.

It has been postulated that it is their high affinity for RNA which allows Morpholinos, and probably PNAs as well, to efficiently invade even quite stable RNA secondary structures,⁴ while the much lower affinity of S-DNAs (cf. Figure 9) necessitates an exhausting search for suitable RNA target sequences.^{7,8} Such searches are probably necessarily extensive because in natural RNAs unstructured regions and regions having secondary structures of only minimal stability are relatively rare. Thus, the widely differing RNA-binding affinities between S-DNAs and the advanced non-ionic Morpholinos and PNAs may account for why Morpholinos, and probably PNAs, typically have an exceptionally high targeting success rate⁹ (on the order of 75% to 85%), while S-DNAs generally have a much poorer targeting success rate^{7,8} (on the order of 10%).

Minimum Inactivating Length (MIL)

A useful measure of an antisense structural type is its 'Minimum Inactivating Length' (MIL), which may be defined as the shortest length of oligo of a given structural type which achieves substantial inhibition of its targeted sequence at a concentration typically achievable within cells. It should be noted that the measured MIL value for a given structural type varies somewhat as a function of sequence, G+C content, and concentration of the oligos tested. Nonetheless, by testing a range of oligo lengths targeted against the same target

Table 3. MIL values for different structural types

Structural type	MIL value
S-DNA	8
PNA	10
Morpholino	15

region, one can obtain reasonable comparative MIL values for various structural types of interest.⁴

Figure 10 shows the results of experiments carried out to estimate MIL values of S-DNAs, PNAs, and Morpholinos in a cell-free translation system (with added RNase H to afford good S-DNA activities). In these activity-versus-length experiments a set of oligos of the three structural types ranging in length from 8 bases to 30 bases were targeted against a region of rabbit alpha-globin leader sequence (Figure 10a), and a second set were targeted against a region of the Hepatitis B virus (HBV) leader sequence (Figure 10b). These two sets of oligos at a concentration of 300nM were assessed in a cell-free translation system for their abilities to inhibit translation of a downstream luciferase-coding sequence. The experimental procedures, oligo sequences, and RNA targets used in this study are detailed elsewhere.^{4,28}

Table 3 gives the approximate MIL values derived from this length-versus-activity study.

As will be discussed in the next section, these differing MIL values strongly influence the preferred applications of PNAs and Morpholinos, particularly in regard to applications in complex systems.

Specificity

The first strategy as regards specificity may be called ‘short-is-good’. A widely accepted criteria for specificity of an antisense oligo is how well it can distinguish between its targeted sequence and a non-targeted sequence differing by only one base. In general, an oligo’s ability to discriminate on the basis of a single base mismatch increases as its length decreases, reaching maximum discrimination at a length corresponding to, or just slightly greater than the MIL value for that structural type. Thus, a PNA of about 10 or 11 subunits in length would be expected to have maximum single-base mis-pairing discrimination for that structural type, while a Morpholino of about 15 or 16 subunits in length would be expected

to exhibit maximal single-base mis-pairing discrimination for that structural type. Since a single base mis-pairing in a 10-mer PNA/RNA duplex (10% of bases mis-paired) has a substantially larger impact on duplex stability than a single base mis-pair in a 15-mer Morpholino/RNA duplex (7% of bases mis-paired), the higher-affinity PNAs provide a substantial advantage over lower-affinity Morpholinos for applications requiring single-base mis-match discrimination. Such applications include targeting single nucleotide polymorphisms (SNPs) and targeting point mutations.

Another strategy is that ‘longer-is-better’. While the challenge of discriminating between a single base difference has received much attention in the antisense field, and is well met by short, high-affinity oligos such as PNAs, for antisense applications in complex systems the principal challenge is quite different. Such mainstream applications include determining the function of newly-sequenced genes; generating morphants^{9,27} in embryos; validating targets in drug development programs; and developing therapeutics for viral diseases and cancers. The challenge in these complex systems is to strongly inhibit a targeted RNA (achieve high efficacy) without inadvertent inhibition of any other RNAs in the system (i.e., to achieve high specificity). Stated differently, what is needed in complex systems is an oligo which affords high efficacy, while also rigorously discriminating between its target RNA and thousands to tens of thousands of non-target RNAs.

To appreciate the challenge of obtaining both high efficacy and high specificity in a complex system such as a human, first consider the extreme case of a hypothetical ultra-high affinity 5-mer modifier-type oligo that has an MIL (Minimum Inactivating Length) of 5. Most RNAs in the pool of RNA transcripts will contain an average of about 4 copies of any given 5-mer sequence, based on an average RNA transcript length of about 4000 bases. Thus, if all sequences in the RNA transcripts were targetable, then one would expect this 5-mer oligo not only to inactivate its desired targeted RNA species, but also to inactivate nearly all other RNA species in the system.

For the case of a blocker-type oligo (such as a PNA or Morpholino), the specificity situation is not quite as bleak. This is because blocker-type oligos are generally only targetable against about 5% of the bases in a typical RNA transcript (cf. Figure 2a). As a consequence, an ultra-high affinity 5-mer blocker oligo is only expected to inadvertently inhibit about 20% of the non-targeted RNAs in the system – but this is still far

too little specificity for most applications in complex systems.

This brings us to what I believe are two crucial design requirements for an antisense oligo suitable for achieving both high efficacy and high specificity in a complex system. First, the oligo's MIL (Minimum Inactivating Length) value must be sufficiently large that the oligo has little chance of inadvertent inactivation of non-targeted species in the system's entire pool of RNA transcripts. Second, in order to achieve high efficacy the oligo's length should be appreciably longer than its MIL.

In regard to a lower limit for the MIL of oligos suitable for use in a complex *in vivo* system, for human use the current estimates are that the pool of RNA transcripts (before splicing) comprise about 30,000 species²⁹. If pre-spliced RNA transcripts average on the order of about 4,000 bases in length, this gives approximately 120 million unique-sequence bases in the RNA pool, of which an estimated 5% (about 6 million bases) are targetable by advanced blocker type oligos (see Figure 2a). Table 4 below gives estimated numbers of RNA species in a human which would be inadvertently inhibited by oligos having the indicated MIL values and lengths corresponding to those values.

The values in Table 4 suggest that in order to have a reasonable chance of no inadvertent targets in a complex system comprising about 6 million bases of targetable unique-sequence RNA, an oligo should have an MIL value of about 12 or greater.

A 12-mer oligo with an MIL of 12 should have excellent specificity in a system as complex as a human; nonetheless, that 12-mer also would have only marginal efficacy. Since increasing binding affinity in order to increase efficacy would decrease the MIL below the value needed for high specificity in this complex system, the remaining option is to increase efficacy by increasing the oligo's length. As for the suitable length for achieving high efficacy, we generally find that Morpholino oligos 25 bases in length can provide high efficacies (90% to 100% target inhibition) at modest concentrations (100 nM to 1000 nM) in cell-free test systems where actual oligo concentration is known.^{3,4,30,31} However, increasing the length of the oligo in order to increase its efficacy also leads to a modest increase in the number of potential inadvertent targets for that oligo. To illustrate, if the MIL for an oligo of a given structural type is about 15 (the case for Morpholinos) and the oligo length is 25, that oligo actually contains 11 different 15-mer sequences, each with its own potential for inadvertent

Table 5. Estimated numbers of inadvertent targets in RNA pool of 6 million targetable bases

MIL value	Estimated number of inadvertent targets for 25-mer oligos
8	1650
9	390
10	92
11	21
12	5.7
13	1.5
14	0.4
15	0.1

inhibition of non-targeted sequences. Thus, when the greater length needed for high efficacy is factored in, estimated numbers of inadvertent targets in the human RNA pool as a function of MIL are calculated as:

Inadvertent targets =

$$(\text{pool complexity} / 4^{\text{MIL}}) (\text{oligo length} - \text{MIL} + 1)$$

Using this equation, estimated numbers of inadvertent targets in the human RNA pool are tabulated for 25-mer oligos as a function of the oligos' MIL values.

The values in Table 5 suggest that to achieve high specificity in a human, a high-efficacy 25-mer should have an MIL of about 14 or greater. Thus, I predict that to achieve both high efficacy and high specificity in a human one should use an oligo structural type with an MIL value of about 14 or greater, and the oligo length should be on the order of 50% to 100% longer than the MIL value. Not unexpectedly, the lower-affinity Morpholinos (MIL ~15) fit these design criteria appreciably better than the higher-affinity PNAs (MIL about 10).

To test this theoretical prediction concerning how to achieve both high efficacy and high specificity in a complex system, we have carried out experiments to assess relative specificity of S-DNA, PNA, and Morpholino oligos in a test designed to emulate a high-complexity system.^{4,28} In these experiments, two oligos of each structural type were used. One oligo of each type was perfectly complementary to its targeted mRNA (globin leader sequence) to provide a measure of total inhibition achieved by that structural type as a function of oligo concentration. The other oligo

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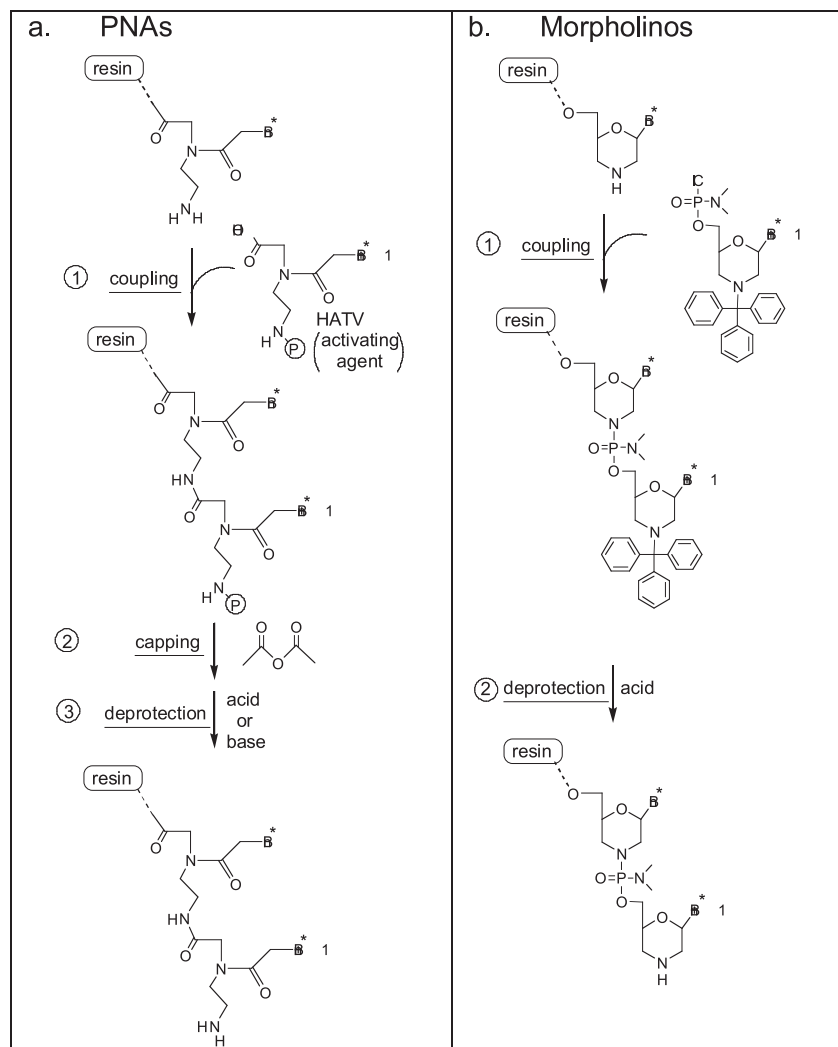


Figure 4. Representative oligo assembly cycles.

Table 4. Estimated numbers of inadvertent targets in RNA pool of 6 million targetable bases

MIL value (X)	Sequence permutations (4^x)	Estimated number of inadvertent targets ($6,000,000 / 4^x$)
8	65,500	92
9	262,000	23
10	1,050,000	6
11	4,190,000	1.4
12	16,800,000	0.4
13	67,100,000	0.09
14	268,000,000	0.02
15	1,070,000,000	0.006

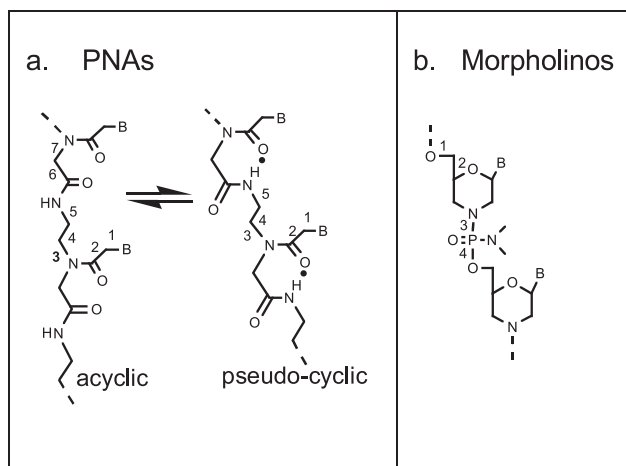


Figure 5. Backbone bonds with relatively free rotation.

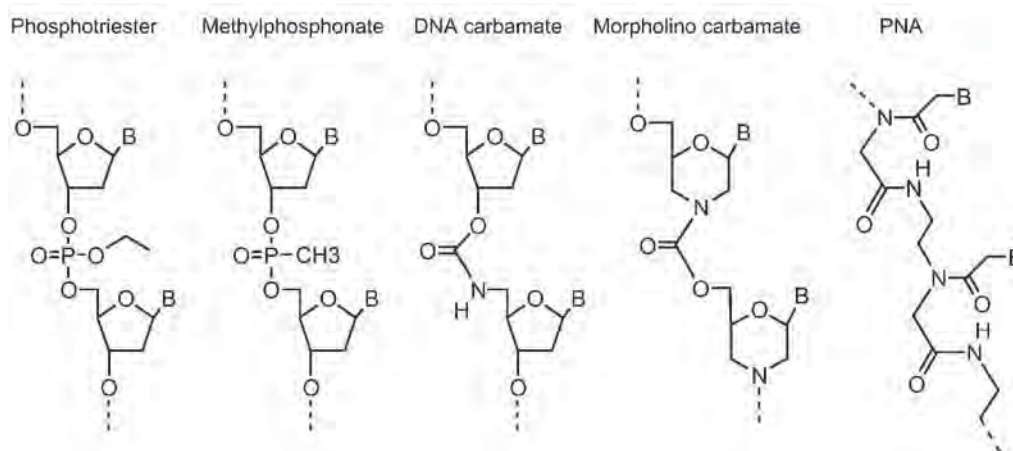


Figure 6. Non-ionic oligos with low aqueous solubilities.

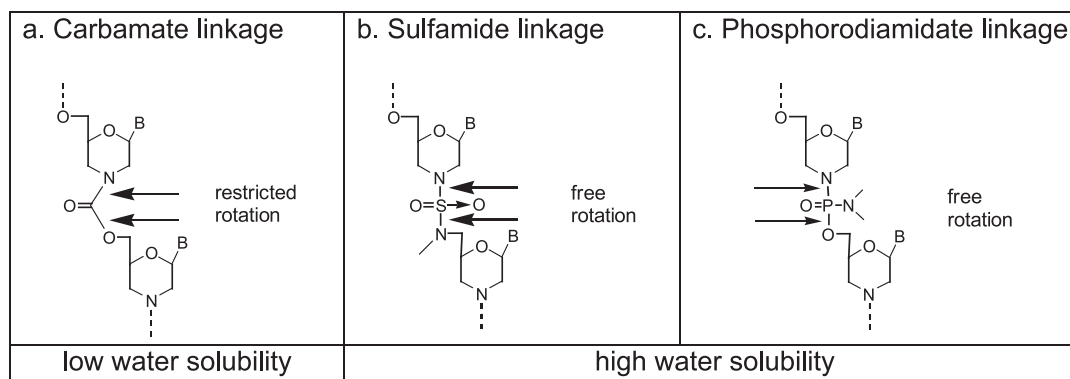


Figure 7. Morpholino oligos with varying aqueous solubilities.

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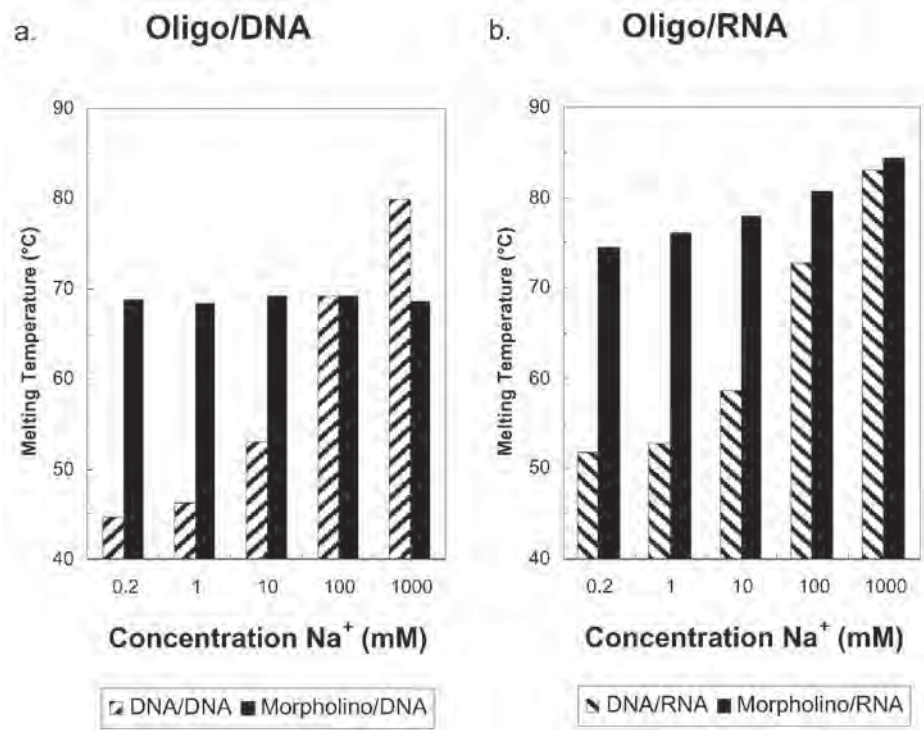


Figure 8. Salt dependence of T_m values for oligo/DNA and oligo/RNA duplexes.

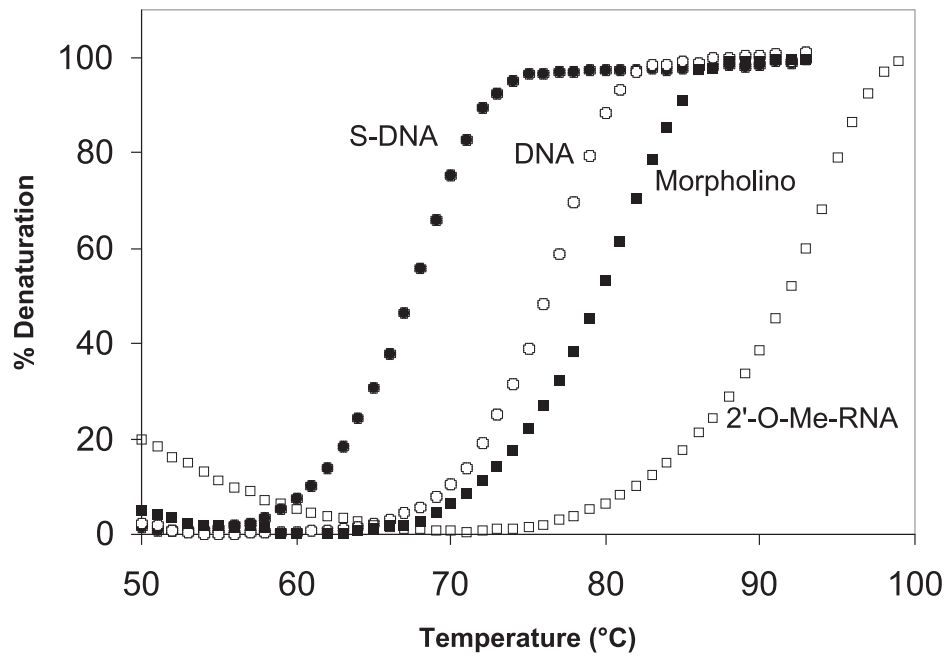


Figure 9. Thermal transitions of 20-mer oligo/RNA duplexes.

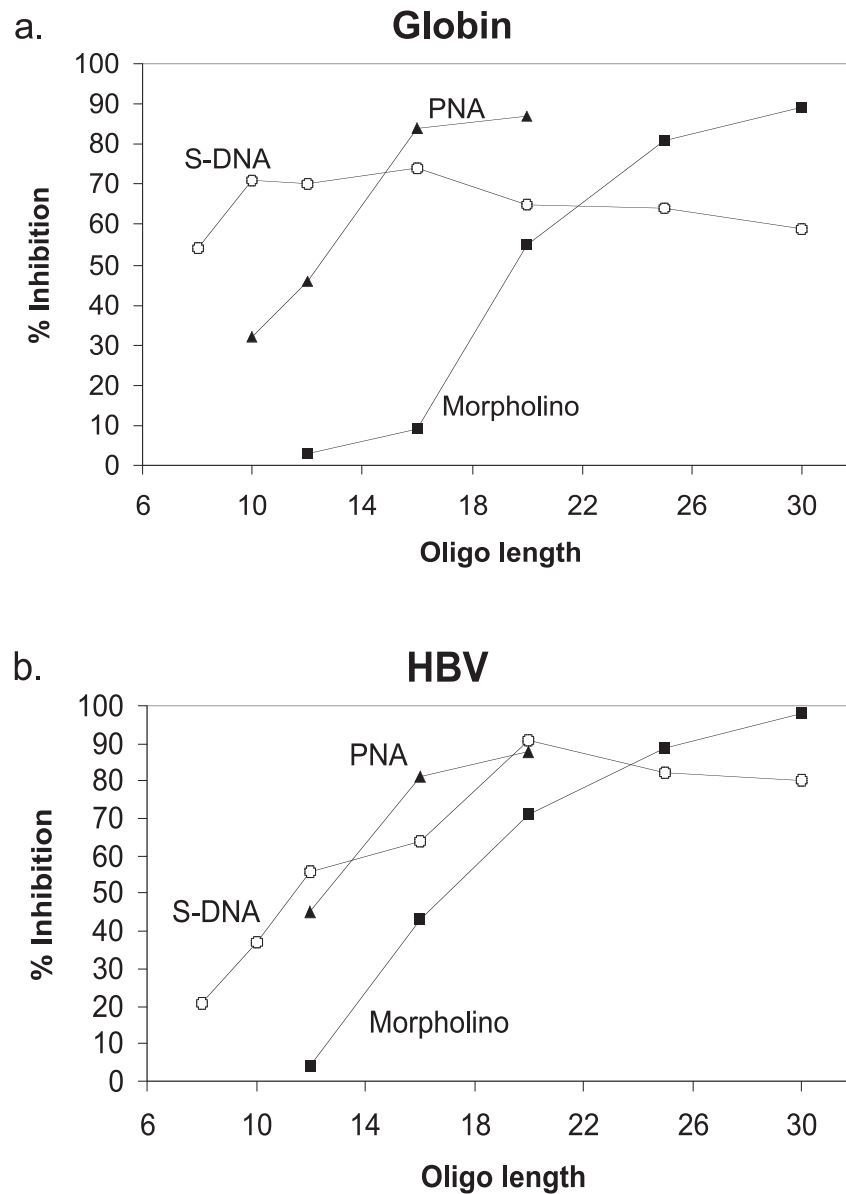


Figure 10. Antisense activity as a function of oligo length.

of that type incorporated mis-pairing to that same target sequence, with the longest run of perfect pairing comprising 10 contiguous base-pairs, to provide a reasonable emulation of the estimated level of sequence homology likely to be encountered in the RNA pool within a representative human cell. For the S-DNA and Morpholino structural types 25-mer oligos

were used and the mis-paired oligos contained four mis-pairs to the target sequence. For the PNA structural type 20-mer oligos were used and the mis-paired oligo contained three mis-pairs.

Specific inhibition was calculated as the difference between the inhibition value for the perfectly-paired oligo and the inhibition value for the mis-paired oligo

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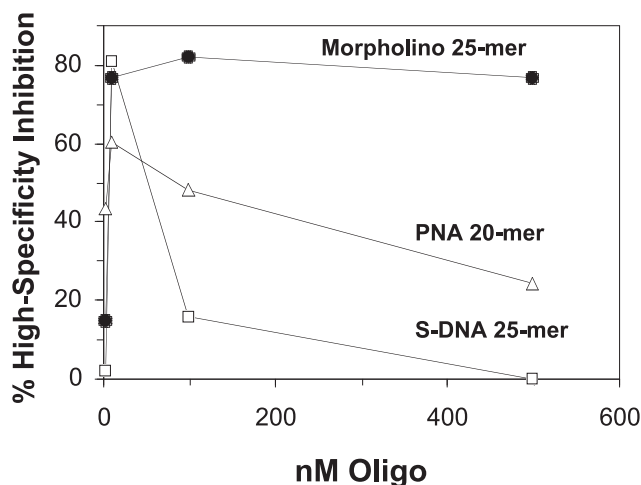


Figure 11. High-specificity component of inhibition by three structural types.

at each concentration. This provides a measure of the high-specificity inhibition achieved by that structural type as a function of concentration. Figure 11 shows a plot of this high-specificity component.

In accord with the length-versus-activity results in Figure 10 and the calculated data in Table 5, the experimental results in Figure 11 demonstrate that in this test emulating a high-complexity system, the high-MIL Morpholinos (MIL ~15) indeed exhibit substantially better sequence specificity over a wider concentration range than the lower-MIL PNAs (MIL about 10), which in turn exhibit substantially better sequence specificity over a wider concentration range than S-DNAs (MIL ~8). These results provide support for Morpholinos being a preferred structural type for applications in complex systems.

Delivery into cultured cells

Until the mid-1980s most antisense experiments were carried out in cell-free test systems where the focus was on assessing prospective structural types for directly inhibiting translation of their targeted mRNAs. By the late 1980s and early 1990s, however, the antisense field had evolved to a stage where experiments were being carried out with cultured cells, at which point serious problems were encountered. Studies by several groups elucidated one particular problem – in cultured cells, neither ionic nor non-ionic antisense oligos can diffuse across cell membranes at any reasonable rate.^{32,33} Instead, much evidence suggests that antisense oligos enter cultured cells via endocytosis and subsequently most or all of the oligos are

degraded, remain trapped in the endosome/lysosome compartment, or are exocytosed back to the extracellular medium.³⁴ Thus, under normal conditions antisense oligos fail to attain entry into the cytosol/nuclear compartment where their targeted sequences reside.

This delivery challenge has led to wide-ranging efforts to develop effective methods for delivering antisense oligos into the proper subcellular compartment of cultured cells. To a large extent these efforts have been successful, though toxicity from the delivery reagents remains a significant limitation for most of the delivery methods. Of particular note, in the past few years a wide range of components have been investigated for delivering PNAs into the cytosol/nuclear compartment of cells^{35–42} and some of these may also hold promise for *in vivo* delivery.

In our own experience, delivery methods which work with Morpholinos generally also work well with PNAs. For example, scrape delivery is one method that is simple, effective and reliable for delivering both PNAs and Morpholinos into adherent cells.^{43,44} It is also one of the few methods which work in the presence of high concentrations of serum.

A new and even more effective method suitable for delivering both Morpholino and PNA oligos entails complexing the non-ionic oligo with a partially complementary DNA oligo and then mixing this partial duplex with the weakly-basic polyamine, ethoxylated polyethyleneimine (EPEI), after which the composite complex is added to cells.⁴⁵ The EPEI, which is only partially ionized at pH 7, serves both to bind electrostatically to the negatively-charged DNA component

of the oligo/DNA duplex, and to bind electrostatically to negatively-charged cell surfaces, effecting rapid endocytosis of the oligo/DNA/EPEI complex. It is believed that when the pH drops within the endosome, the EPEI is further ionized to the point where its charge density is sufficient to permeabilize the endosomal membrane, allowing release of the oligo into the cytosol of the cell. This 'Special Delivery' method (see also: www.gene-tools.com) is effective with a broad range of cell types, is quite efficient with both adherent and non-adherent cell types, and is less damaging to cells compared to most other delivery methods. Regrettably, the method does not work well in the presence of high concentrations of serum.

Delivery in vivo?

While methods for delivering antisense oligos to the cytosol/nuclear compartment of cultured cells are now fairly well developed and reliable, most or all of those methods appear to be ineffective and/or too toxic for use *in vivo*. In light of these limitations in applying successful cultured-cell delivery methods *in vivo*, it came as a considerable surprise to many in the antisense field when reports began to circulate in the mid-1990s that by some as-yet-undefined mechanism, antisense oligos are able to gain entry into the cytosol/nuclear compartment of cells *in vivo*,^{46,47} particularly in the liver and kidney. Subsequent to these reports, there have been additional reports implying successful *in vivo* delivery. In this context, it is widely touted that one S-DNA oligo (Vitravene) has been approved by the Food and Drug Administration of the USA for *in vivo* therapeutic application in humans,⁴⁸ and this is commonly construed to be definitive proof that antisense oligos (or at least S-DNAs) readily gain entry into the cytosol/nuclear compartment of cells *in vivo*. However, this 'proof' of effective cytosol/nuclear delivery *in vivo* is less impressive than it may appear, particularly in light of evidence that this particular S-DNA (ISIS 2922) does not function by an antisense mechanism within cells, but instead probably functions largely or solely in the extracellular milieu as an immune stimulatory agent due to CpG-containing sequences at each end of the oligo.⁴⁹

Further evidence for effective cytosol/nuclear delivery *in vivo* has come from reports from AVI BioPharma. This company developed a Morpholino oligo (Resten NG) which was reported to be effective in inhibiting restenosis following balloon angioplasty.^{50,51} However, it should be appreciated that in this particu-

lar application, connections between cells in the artery wall are seriously perturbed during the course of the angioplasty procedure, and such perturbations have been reported to permeabilize plasma membranes of cells in the artery wall for a significant period of time following the scraping procedure.⁵² It is believed that such cell permeabilization in the artery wall probably occurs by a mechanism similar to that which allows rapid cytosolic entry of antisense oligos during scrape delivery of cultured cells.⁴³ The key point here is that while effective delivery into the proper subcellular compartment may be achieved in AVI BioPharma's particular *in vivo* application, nonetheless, this does not imply that effective cytosol/nuclear delivery will also occur equally well in other *in vivo* applications where the cells are not mechanically perturbed.

Still another increasingly popular antisense application which provides evidence for effective cytosol/nuclear delivery *in vivo* is the successful use of Morpholinos for generating morphants (i.e., antisense-mediated morphological changes which mimic mutational changes) in early-stage embryos of sea urchins, frogs, and zebra fish.^{9,27} Again, appearances may be deceiving in regard to delivery, at least in the case of zebrafish. This is because the antisense oligos are injected into the zebrafish eggs before or very shortly after fertilization, at which time the normal permeability barriers between embryonic cells have not yet formed. Only somewhat after the 32-cell stage in zebrafish embryos do the normal permeability barriers begin to form between cells of the developing embryo.^{53,54} Thus, when antisense oligos are injected into such eggs or embryos just beginning to undergo cell divisions, those antisense oligos have full access to all cells of the organism, and will reside and function within all those cells through multiple cell divisions.¹⁶ Preliminary results from collaborators at the University of Oregon suggest that when antisense oligos are instead injected into the yolk or the vascular compartment of later stage embryos wherein the normal cellular permeability barriers have formed, those oligos fail to generate the expected phenotypic changes, suggesting a lack of reliable *in vivo* delivery.

Further evidence suggesting that Morpholino oligos do not readily cross cell membranes *in vivo* comes from studies with frog eggs/embryos, whose cells exhibit normal permeability barriers immediately after the first cell division. In this system, when fluorescent-tagged Morpholinos are injected into one cell of a 2-cell stage frog blastomere, the oligos remain only in the direct descendents of that injected cell (one side

of the developing embryo), at least through the free-swimming tadpole state (stage 43). The same basic result was also found in a functional test wherein the Morpholino was targeted against a stably-integrated transgene expressing green fluorescent protein.¹⁶

Thus, while many scientists in the antisense field contend that antisense oligos readily enter the proper subcellular compartment of many or all cells *in vivo*, many remain skeptical of most claims for antisense activity *in vivo*, at least in those cases where no delivery mechanism is apparent and no delivery component was used to achieve entry into the cytosol/nuclear compartment of the cells.

There are a number of important reasons for continued skepticism concerning *in vivo* delivery of bare antisense oligos:

1. One would expect that cells *in vivo* should not be significantly more permeable than cells in culture to large polar molecules such as antisense oligos, and this expectation appears to be supported by multiple studies with vertebrate embryos. For instance, preliminary studies indicate that a Morpholino oligo which generates a distinctive phenotypic change (fluorescent blood) when injected into early-stage zebrafish embryos fails to generate that same phenotype when injected into later-stage embryos wherein normal permeability barriers have formed. Further, Morpholino oligos injected into one cell of a 2-cell stage frog blastomere remain on one side of the frog embryo through the free-swimming tadpole stage.¹⁶

2. Early attempts to use 'naked' antisense oligos to inhibit virus infections in mice and other model organisms, where a definitive answer would be expected if the oligos were effective and high levels of *in vivo* delivery occurred, appear to have been relatively ineffective.

3. In most reports of *in vivo* antisense activity, S-DNAs were utilized, which are well known to generate multiple non-antisense effects due to their actions in the extracellular medium and at cell surfaces.^{55,56} It is noteworthy that several of those non-antisense effects have been shown to be sequence dependent – which can lead to erroneous conclusions even when apparently valid control oligos are used in the experiments.

4. Experiments targeting cellular genes in whole animals inherently entail many more uncertainties than targeting defined marker genes transfected into cultured cells, and results *in vivo* are often indirect and more open to interpretation. These greater uncertainties and less direct results in animal studies raise the chances that positive results are in reality just normal

statistical fluctuations, flaws in experimental design, or any of a host of other problems common to complex experimental systems.

5. It appears unlikely that all of the major pharmaceutical companies, who together invested billions of dollars in antisense research in the late 1980s and early 1990s, would have abandoned (in the late 1990s) their attempts to develop antisense therapeutics if they had been able to repeat the claimed *in vivo* successes of the small biotech antisense companies.

Notwithstanding this somewhat pessimistic assessment, I would like to conclude this section with two positive statements regarding *in vivo* delivery:

First, I believe that achieving safe and effective delivery of antisense oligos into the cytosol/nuclear compartment of a wide variety of cell types *in vivo* probably constitutes the last major challenge which must be met in order for antisense oligos to fulfill their great promise of safe and effective therapeutics for a broad range of viral diseases, cancers, possibly autoimmune diseases, and a host of other currently intractable disease states.

Second, based on recent advances by several research groups including GeneTools, it appears likely that this delivery challenge will be met soon, perhaps within the coming five years.

Applications

Research tools

PNAs have been used for an unusually wide variety of novel research applications. Probably because of their exceptionally high binding affinity and unusually flexible backbones, PNAs have proven particularly useful for forming triplexes with DNA and RNA. With duplex DNA targets, in low salt conditions (to destabilize the DNA/DNA duplex) an all-pyrimidine PNA can invade a targeted purine or pyrimidine stretch of duplex DNA to form a PNA/DNA/PNA triplex and a single-stranded displacement loop comprising the pyrimidine-containing strand of DNA.¹⁷ For the case of single-stranded, all-purine RNA targets, PNAs also can form ultra-stable PNA/RNA/PNA triplexes which have the unique capability of blocking translocation of ribosomes – even in downstream amino acid-coding regions of a targeted mRNA. Thus, for these rare target sequences, such specially-designed PNAs can be effective in a region of the mRNA which is normally only targetable by modifier-type antisense structural types.⁶

Because of their exceptionally high binding affinity, PNAs also excel in targeting inherently short RNA sequences, such as the short RNA sequence exposed in the telomerase enzyme.^{57,58}

A particularly promising exploitation of high affinity PNAs is their use as antisense antibacterials, where the limited porosity of bacterial cell walls largely preclude use of antisense oligos longer than about 12 to 14 bases. A major advance in this application was recently reported wherein addition of a short cell wall-permeabilizing peptide to a 12-mer PNA dramatically increased the PNAs antibacterial efficacy.⁵⁹

As noted in earlier sections, PNAs are also attractive because of their compatibility with peptide synthesis conditions. This allows one to synthesize a PNA, and then while the PNA is still protected and on its synthesis resin, amino acids are added stepwise to form peptide adducts suitable for enhancing delivery into cells^{35,60} or for other applications such as diagnostics.

PNAs have been used in a novel extra-cellular therapeutics application wherein an antibody/PNA adduct is used to bind to cancer-specific antigens on the surface of cancer cells *in vivo*. This is followed by addition of a second complementary PNA to which is attached a suitable radioisotope. The isotope-carrying PNA then rapidly pairs to its complementary PNA linked to the antibody bound to the cancer cells. This process effectively concentrates the isotope in proximity to the cancer cells – thereby substantially increasing the damage to the cancer cells and decreasing damage to the non-cancer cells.⁶¹

PNAs also have been investigated for use in a variety of experimental systems for detecting and quantitating human or animal genetic sequences.^{62–66}

In contrast to the wide-ranging applications of PNAs, so far Morpholinos have been used primarily for classical antisense applications in complex systems. Such applications include correcting splicing errors in pre-mRNAs in cultured cells^{44,45} and in extra-corporal treatment of cells from thalassemic patients.⁶⁷

Morpholinos are often used for classic antisense inhibition of targeted mRNAs in cultured cells when both high efficacy and high specificity are desired.^{68–70} In this context, a Morpholino targeted against the C-myc mRNA is in Phase 3 clinical trials for prevention of restenosis following balloon angioplasty.⁵⁰ In this application it is likely that delivery is achieved *in vivo* via scrape delivery into cells of the artery wall during the angioplasty procedure.

Perhaps the most demanding application of Morpholinos is in developmental biology.^{9,27,71–74} For this application it is necessary to inject a very small volume of a high concentration of oligo into eggs or early-stage embryos⁹ or to electroporate oligos into specific tissues in later-stage embryos.⁷⁴ Obviously, oligos for this application must be very soluble in water (i.e., multi-millimolar). Such oligos also must exhibit extremely high sequence specificity and have negligible toxicity and negligible non-antisense effects. The need for exquisite specificity is because within a brief period of time (a few days) the rapidly developing organism expresses most of its entire set of genes and so the antisense oligo must efficiently inhibit its targeted mRNA without significantly affecting any of the very large number of other mRNA species which are present at one time or another during embryogenesis (but mostly absent from terminally differentiated cells). Developmental biology applications also require that the oligos be stable in biological systems for long periods of time. To the best of our knowledge, to date Morpholinos are the only antisense type shown to work predictably, specifically and without toxicity in this very demanding application.

A particularly interesting application in developmental biology is the use of Morpholinos to selectively target zygotic RNAs without concomitant inhibition of maternal RNAs coded by the same gene.⁷⁵ This is achieved by targeting intron/exon splice junctions, which are present in the newly transcribed zygotic pre-mRNAs, but absent from the already-spliced maternal mRNAs.

Another valuable strategy which can be utilized in studies with frog eggs is to inject the test Morpholino oligo into only one cell of a 2-cell stage blastomere. The other cell of the blastomere is either not injected or is injected with a control Morpholino oligo. In the course of development, all cells on one side of the embryo come from the injected cell and all cells from the other side of the embryo come from the non-injected or control-injected cell. It has been shown that in this manner one side of the embryo serves as an excellent control for the other side because Morpholino oligos in cells of one side of the embryo cannot pass over to cells of the other side of the embryo.¹⁶

Clinical diagnostics

For the past few decades, it has appeared to many scientists involved in nucleic acid research that nucleic acid probes should be greatly superior to antibodies

for detecting infectious diseases in the clinic. This is because nucleic acid probes (or other probes) can have higher binding affinities than antibodies as well as greater specificity than even monoclonal antibodies. Most importantly, generating a probe specific for a selected genetic sequence (analyte strand) is much simpler, faster, and more reliable than generating a corresponding pathogen-specific antibody. In spite of these apparent advantages, after several decades of development, probe diagnostics have only made minor inroads into the clinical diagnostics arena, with antibody-based diagnostics still enjoying nearly complete dominance.

There are three principal challenges which may be responsible for classic probe diagnostics having failed to gain a significant foothold in the clinic: 1. Stringency. Probes generally require precise control over stringency (i.e., salt, denaturant, temperature) during probe/target pairing, giving false positives if stringency is too low and false negatives if stringency is too high. This raises the specter of poor reliability or reproducibility, which can be catastrophic in the context of clinical diagnostics.

2. Speed. For samples containing low concentrations of analyte strands, for example ~600 virus particles per ml of blood, which corresponds to about 1 attomolar, pairing of probe to its complementary analyte strand typically requires many hours, or a relatively complex time-consuming, expensive, error-prone pre-amplification of a key portion of the analyte strand by polymerase chain reaction (PCR) or some analogous target amplification procedure.

3. Sensitivity. The concentration of analyte strands (e.g., viral genetic sequences) in clinical samples are often in the zeptomolar (10^{-21}) or attomolar (10^{-18}) range and probe diagnostic methods for detecting these very low analyte concentrations generally require complicated time-consuming and expensive pre-amplification of a portion of the analyte strand (such as by PCR) or use of complicated and expensive detection equipment not appropriate for a clinical setting.

One major exception to the limited sensitivity of probe diagnostics systems is the Branched DNA system developed at Chiron and now widely used in the clinic for quantitation of HIV.⁷⁶ Still, even the Branched DNA system is slow, complicated, and expensive compared to most antibody-based diagnostics.

High-affinity non-ionic probes, such as PNAs and Morpholinos, are well-suited to overcoming these

many challenges currently impeding the use of probe diagnostics in most clinical applications.

With respect to stringency, the reason precise control of stringency is important with standard nucleic acid probes is that under low stringency conditions (i.e., too much salt or too little denaturant or too low of a temperature) the target sequence of the analyte strand, complementary to the probe, is largely unavailable for pairing to the probe because of extensive secondary structure in the analyte strand. Conversely, when the stringency is too high (i.e., too little salt or too much denaturant or too high of a temperature) the probe is unable to stably bind to its target sequence in the analyte strand.

In contrast to the case for standard ionic probes (e.g., DNA and RNA), when using a non-ionic probe and pairing in salt-free water, essentially all secondary structure in the analyte strand is disrupted due to electrostatic repulsion between backbones, allowing full access of the probe to its target sequence in the analyte strand. Further, because the probe has no backbone charge, these same salt-free conditions have little or no impact on the probe/target pairing (see Figure 8 and Reference 1). Thus, by using a non-ionic probe (PNA or Morpholino) the challenge of precisely controlling stringency can be disposed of simply by carrying out the pairing step in salt-free water at a temperature anywhere in a fairly large range (ambient to about 50 °C). As a consequence, use of non-ionic probes can significantly simplify the diagnostic system. More important, it can also greatly reduce the chance of false negatives and false positives.

With respect to speed, a method has been developed which allows very rapid pairing (seconds to a few minutes) between a probe and its target sequence in an analyte strand, even when that analyte strand is present at extremely low concentrations (zeptomolar) in a biological sample.⁷⁷ In one embodiment of that method a surface (e.g., microbeads or a porous frit) is derivatized with both a weakly-basic oligoamine (effective pKa ~6) and a non-ionic probe. When a biological sample buffered at about pH 5 (to assure ionization of the oligoamine) is contacted with that oligoamine/probe surface, all polyanionic nucleic acids, including any analyte strands, are adsorbed in seconds to that oligoamine/probe surface via electrostatic bonds between the cationic oligoamines and the anionic nucleic acids. The surface is then washed with salt-free water to disrupt secondary structures in the analyte strand and allow pairing between surface-bound probes and analyte strands held in immediate

proximity to the probe due to electrostatic bonding to the interspersed oligoamines. This probe/target pairing is generally complete within a couple of minutes, even when the analyte strand was originally present at extremely low concentrations. The surface is next washed with pH 8 buffer to deionize the weakly basic oligoamines and thereby terminate the electrostatic bonding between the surface-bound oligoamines and nucleic acids. Washing the surface with pH 8 buffer effects removal of all nucleic acids except analyte strands still linked to the surface via Watson/Crick bonds between the surface-bound probes and target sequences of the analyte strands.

To summarize this rapid-pairing strategy, biological sample buffered at pH 5 is contacted with the oligoamine/probe surface to capture all nucleic acids. The surface is then washed with water to allow pairing between the probe and target sequences within any analyte strands. Several minutes later the surface is washed with pH 8 buffer to remove all non-analyte strands. By this means, pairing which would normally take many hours is achieved near quantitatively in several minutes.

In regard to sensitivity, in the research laboratory low-copy-number analyte strands are routinely detected either by selectively pre-amplifying a portion of the analyte strand (such as by PCR) or by highly amplifying a signal associated with the probe – where said amplification is generally complicated, labor intensive, and expensive. While these complexities and costs may be acceptable in a research setting, I believe they constitute a major impediment to use of probe diagnostics in most clinical applications.

Thus, it appears that the last major challenge in developing probe diagnostics suitable for the clinic is the development of a direct detection scheme which is capable of reliably detecting as few as about ten to a hundred analyte strands in a 1–5 ml biological sample, and which is also fast, simple, and cheap. While such a simple high-sensitivity direct detection capability appears not to be available at this time, GENE TOOLS is embarking on development of a direct detection method using novel structures and a unique scheme which exploits the special properties of non-ionic probes. This detection scheme actually comprises two key aspects, as follows: 1) a component effective to provide a very large signal (tens of thousands of fluorophores) per analyte strand; and, 2) a mechanism for dramatically reducing the typical level of background signal.

In light of past progress and expected upcoming developments, it is possible that probe diagnostics will finally win a major share of the clinical diagnostics market in the foreseeable future. I further believe that because of the compelling advantages afforded by non-ionic probes, the probe components in those clinical diagnostics will almost surely have non-ionic backbones.

Therapeutics

It should be appreciated that both PNAs and Morpholinos are relatively new structural types, with the currently preferred embodiments only about a decade old. Furthermore, the rate of their development was substantially slowed during much of the past decade because until recently most of the economic resources available in the antisense field from government, the pharmaceutical industry, and investors have been focused on the seriously flawed S-DNAs.

In spite of the above factors, *in vivo* studies with bare PNAs and bare Morpholinos (i.e., oligos with no added delivery component) are now being undertaken by a number of groups – and results from at least some of these studies suggest that I might be mistaken in my belief that a delivery component will generally be required for effective delivery of antisense oligos into the cytosol/nuclear compartment of cells of living animals.

One study providing support for effectiveness of bare PNAs *in vivo* utilized a PNA targeted against the neurotensin receptor 1 and another PNA targeted against the opioid mu receptor. These bare PNAs were injected into the periaqueductal gray region in brains of rats. Each PNA produced the physiological response expected if it had down regulated its targeted mRNA in neuronal cells, and subsequent biochemical assessment of the brains of the treated rats indicated that the concentrations of the protein products coded by the targeted mRNAs were significantly reduced, as would be expected from an antisense mechanism.⁷⁸

In regard to *in vivo* use of bare Morpholinos, AVI BioPharma, owner of the patents covering Morpholinos, is aggressively pursuing the development of a number of different Morpholino therapeutics. To date, none of these prospective Morpholino therapeutics contain a delivery component. In spite of this, AVI BioPharma and their multiple collaborating groups have reported positive results from studies in a variety of different animal species and in humans for Mor-

pholinos targeted against the mRNAs of a number of different genes.^{79–82}

If the foregoing results are valid, and not just special cases of exceptional cell permeabilities in such sites as the brain and liver, then in light of the impressive properties of these advanced antisense structural types, it seems possible that in the future safe and effective antisense therapeutics (Morpholinos and/or PNAs) will be introduced for the treatment of a wide range of diseases.

Conversely, if the *in vivo* results with bare PNAs and bare Morpholinos prove to be invalid or merely special cases, then the introduction of a broad range of antisense therapeutics will likely be delayed until after safe and effective delivery components are developed.

Editor's note: As the inventor and commercial producer of Morpholinos, Dr. Summerton has disclosed his financial interest in Morpholino technology. Also, Dr. Summerton wishes to state that his first-hand experience with PNAs has been limited to experimental comparisons between Morpholinos, PNAs, 2'-O-methyl RNAs, and S-DNAs with respect to properties expected to be important for diagnostic applications and for therapeutic applications against viral diseases and cancers.

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EXHIBIT 28

Gene therapy for Duchenne muscular dystrophy

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Gene therapy has great potential to treat Duchenne muscular dystrophy. Among many proposed strategies to deliver a therapeutic gene to muscle, recombinant adeno-associated virus-mediated gene transfer is the most promising. The recent isolation of new adeno-associated virus serotypes from human and nonhuman primates provides the opportunity to develop vectors that can achieve the long-term expression of a therapeutic gene in muscles of the entire body without detrimental effects. To translate the results from small animal models to clinical trials in humans, further work using larger animal models, such as dystrophic dogs or nonhuman primates, is required. This review also discusses recent progress in other gene transfer-related therapeutic approaches, including targeted exon skipping and gene correction.

Duchenne muscular dystrophy (DMD), which affects one in 3300 males, is a devastating, progressive, muscle-wasting disease caused by mutations in the dystrophin gene [1,2]. Skeletal muscles in DMD are characterized by myofiber degeneration and progressive fibrous and fatty changes. There is, currently, no way to prevent muscle fiber necrosis and patients suffer severely from respiratory and cardiac complications in the second decade of life. The *DMD* gene is among the largest genes known, spanning 2.4 Mb at Xp21 and encoding a 427-kDa subsarcolemmal cytoskeletal protein, dystrophin, and several shorter isoforms (Dp260, Dp140, Dp116 and Dp71). The full-length dystrophin protein is composed of four domains: an N-terminal actin-binding domain, a central rod domain consisting of 24 spectrin-like repeats, a cysteine-rich domain and a C-terminal domain. Dystrophin binds actin at the N-terminal domain, β -dystroglycan at the cysteine-rich domain and dystrobrevin and syntrophins at the C-terminal domain, forming the dystrophin-glycoprotein complex (DGC) at the sarcolemma (Figure 1) [3]. A lack of dystrophin at the sarcolemma causes secondary loss of the DGC and other functional molecules, such as neuronal nitric oxide synthase (nNOS) [4] and aquaporin-4 [5]. Importantly, mutations in the genes encoding other members of the DGC cause several different types of muscular dystrophy. The mechanism of the degeneration and death of dystrophin-deficient myofibers is not yet fully understood, but it is believed that myofibers lacking dystrophin and the DGC at the cytoplasmic membrane are mechanically weak and highly susceptible to contraction-induced injury. As a

result, the affected muscle experiences continuous cycles of myofiber death and regeneration, resulting in the gradual loss of myofibers and contractile force. In addition to mechanical weakness, abnormalities in calcium handling and changes in mitogen-activated protein (MAP) kinase and GTPase signaling in dystrophin-deficient muscle have been reported and proposed as underlying processes of muscular dystrophy [6,7].

At present, only corticosteroids are reported to effectively attenuate the progress of the disease [8], and current treatment options focus on respiratory and cardiac assistance and improvement of quality of life. Many research groups are still attempting to develop an effective therapy for DMD. In this review, we describe recent progress in gene and related therapies for DMD.

Recombinant adeno-associated virus vector: a promising tool for delivery of dystrophin gene to skeletal muscles

Among several gene transfer vectors and methods developed to date, the adeno-associated virus (AAV) vector is the most suitable to introduce the exogenous gene into postmitotic, nondividing myofibers. An AAV is a tiny, nonpathological, replication-defective virus, with a 4.7-kb single-stranded DNA genome, belonging to the parvovirus family. AAV vectors induce fewer immunological and inflammatory responses *in vivo* than adenovirus vectors [9]. Although the virus genome persists predominantly in episomal form, expression of the transferred gene lasts months to years in adult skeletal muscle. To date, more than 100 AAVs with distinct virus genome sequences have been isolated from humans, nonhuman primates and other species [10]. They display varying

Keywords: adeno-associated virus vector, Duchenne muscular dystrophy, dystrophin, exon skipping, gene therapy

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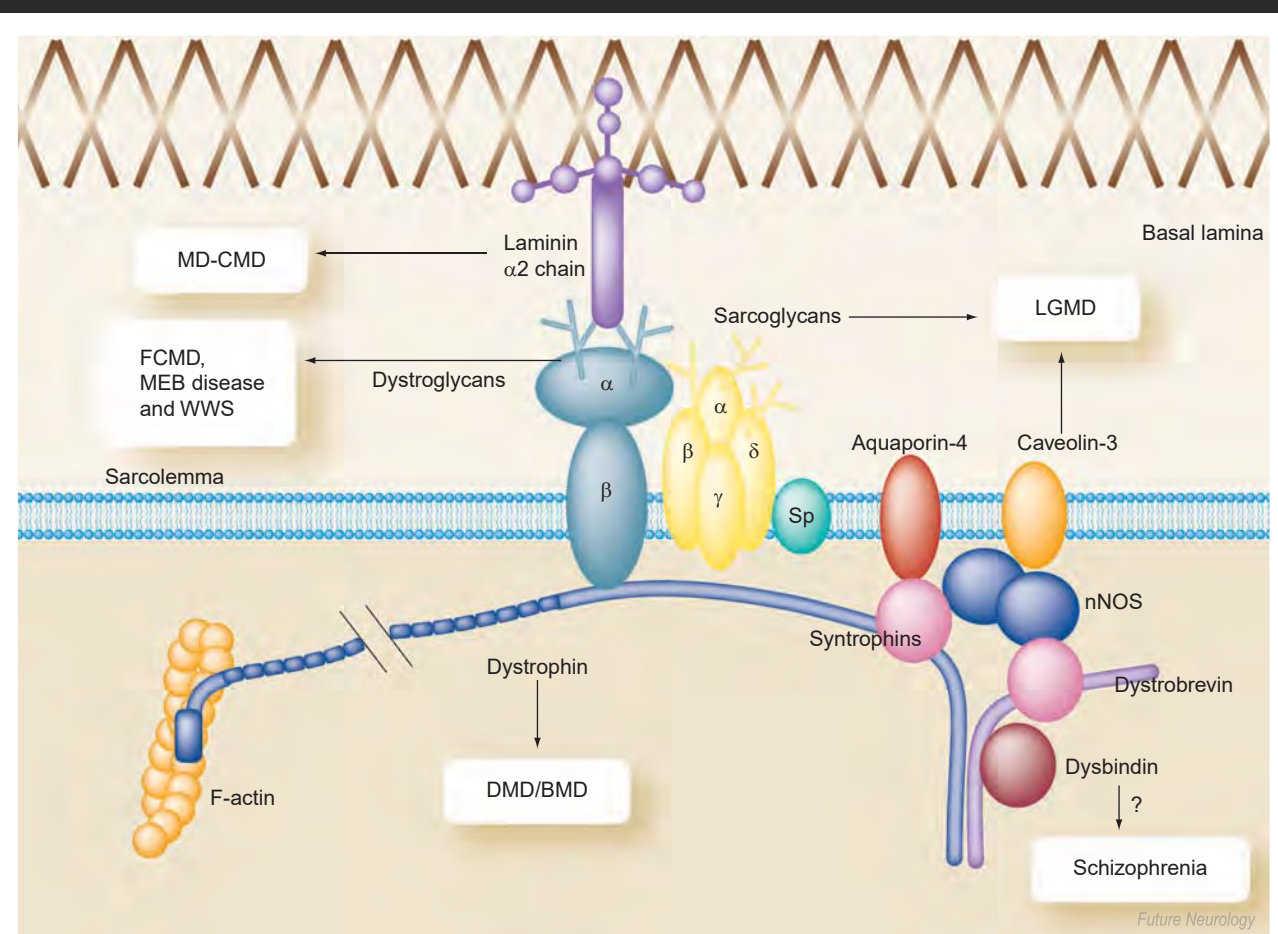
degrees of similarity in their capsid proteins and show diverse tissue tropisms. More than nine AAV vectors have already been developed and evaluated in animal models as a tool for gene transfer *in vivo* (Table 1) [10]. Although the molecular mechanisms of tissue and cell tropisms of AAV vectors are not fully explained, they are likely to use different cellular receptors for entry into and binding to the host cells. The expression of the therapeutic genes is not permanent, mainly because recombinant (r)AAV does not replicate in the host and is barely incorporated into the genome of satellite cells. They are diluted out with the turnover of myofibers and, therefore, repeated

administrations are required. New AAV serotypes would provide good options for follow-up treatments because they have the potential to evade pre-existing neutralizing antibodies against the previously used AAV serotype. However, to avoid the risks of *in vivo* vector delivery, it is important to better understand the vectors and the natural infection with the corresponding virus.

Generation of microdystrophin suitable for use in rAAV vectors

The rAAV vector is a promising tool for gene transfer to DMD muscle, but the limitation of the insertion size to 4.9 kb excludes incorporation of

Figure 1. Dystrophin forms the large dystrophin–glycoprotein complex at the sarcolemma, linking the basal lamina to the cytoskeletal actin.



Mutations in the dystrophin gene end in the secondary loss of dystrophin–glycoprotein complex and other functional molecules, such as nNOS and aquaporin-4. Mutation in the laminin α2 chain gene causes congenital MD. Abnormal glycosylation of α-dystroglycan is commonly observed in FCMD, MEB disease and WWS. Abnormal glycosylation of α-dystroglycan also causes abnormalities in the eye and the CNS. Mutations in any of four sarcoglycan genes (α, β, γ and δ) result in LGMD. These observations emphasize the importance of dystrophin and associated molecules for muscle integrity. The dysbindin (*DTNBP1*) gene is one of the several putative susceptibility genes for schizophrenia.

BMD: Becker MD; DMD: Duchenne MD; FCMD: Fukuyama-type congenital MD; LGMD: Limb girdle MD; MD: Muscular dystrophy; MD-CMD: Merosin-deficient congenital MD; MEB: Muscle-eye-brain; nNOS: Neuronal nitric oxide synthase; WWS: Walker-Warburg syndrome.

Table 1. Characterization of nine serotypes of AAV vectors.

Serotype	Amino acid homology to AAV2 (%)	Isolated from	Tissue tropism				Delivery system
			Skeletal muscle	Heart	Liver	CNS	
1	84	NHP	+++	++	+	+	Local
2	100	Human	+	+	+	+	Local
3	88	Human		+	±	+	Local
4	64	NHP		+	±	+	Local
5	61	Human	+	+	++	++	Local
6	84	Human	++	++	+	+	Local, systemic
7	83	NHP	+++	+++	++	++	Local, systemic
8	84	NHP	+++	+++	+++	++	Local, systemic
9	83	Human	+++	+++	+++	++	Local, systemic

AAV: Adeno-associated virus; NHP: Nonhuman primate.

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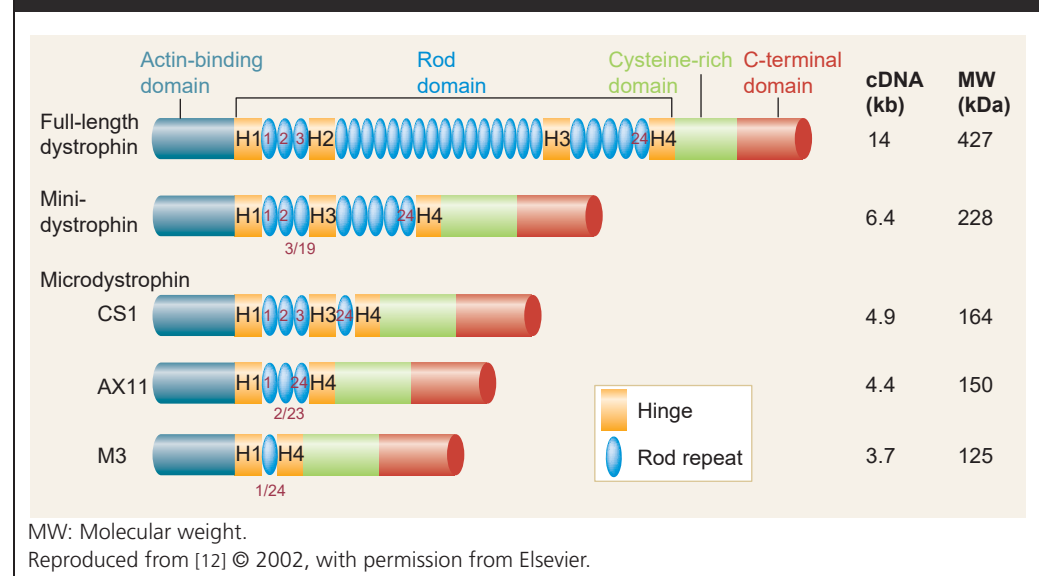
a full-length dystrophin gene (14-kb mRNA, 11-kb open reading frame). To overcome this drawback, several groups have designed small dystrophins in which the long, central rod domains are largely deleted, and tested their functions in dystrophin-deficient *mdx* mice [11]. The functions of three types of microdystrophins (CS1, AX11 and M3; Figure 2) have been tested on microdystrophin-transgenic, dystrophin-deficient *mdx* mice, and it was observed that over-expressed CS1 with four repeats and three hinges almost completely ameliorated dystrophic phenotypes [12]. Therefore, a rAAV2 vector was constructed expressing CS1, driven by a skeletal muscle-specific muscle creatine kinase promoter [13], and was injected it into the

anterior tibialis muscles of immunocompetent adult *mdx* mice. A total of 24 weeks after injection, 50% of myofibers, on average, expressed microdystrophin and the treated muscles demonstrated improved contractile force [14].

Systemic delivery of rAAV-serotype 6, 8 & 9 vectors

Systemic delivery systems for the treatment of DMD require improvement to enable transfer of the therapeutic genes to the complete musculature of the body, especially to the heart and diaphragm. Gregorevic and colleagues reported that intravenous injection of rAAV6 vectors efficiently delivered a microdystrophin gene to the

Figure 2. Structure of full-length dystrophin and constructs of mini- and microdystrophin (CS1, AX11 and M3).



muscles of an adult mouse and the ratio of microdystrophin-positive fibers was increased when co-injected with vascular endothelial growth factor [15]. The widespread expression of microdystrophin was sufficient to correct susceptibility to contraction-induced injury and to lower serum creatine kinase levels [15]. Wang and colleagues, and Nakai and colleagues, demonstrated that AAV8 was more efficient than AAV6 or AAV1 at attaining systemic gene transfer, especially to the cardiac muscles of mice or hamsters, without pharmacological intervention [16,17]. More recently, Inagaki and colleagues reported that AAV9 vectors demonstrated robust systemic transduction in mice [18]. Remarkably, rAAV9 is superior to rAAV8 for gene delivery to cardiac muscle by systemic vector administration [18]. The molecular basis of the high transduction efficiency via the bloodstream is not fully understood, but these results are encouraging for researchers who are developing gene therapies for DMD patients. On the other hand, however, AAV8 or 9 vectors also increase transduction of nonmuscle tissues, such as liver (Table 1), which may be deleterious.

AAV vectors for human muscle

Animal models are indispensable for the evaluation of the efficacy and safety of AAV-mediated gene therapy of DMD, but a recent report on clinical gene transfer studies for hemophilia B demonstrated that the data obtained in preclinical studies in animals are not always predictive of vector efficacy in humans [19]. Certain human populations are exposed to AAVs in daily life: 50–96% are seropositive for AAV2 and at least a third have a neutralizing antibody to AAV2 [10]. Therefore, prior exposure to AAV2 explains the unsatisfactory results of clinical trials using rAAV2-factor IX gene transfer on hemophilia B patients [19]. The new serotypes of AAVs are reported to be prevalent in human and non-human primates. Prescreening of patients for neutralizing antibodies against the vector serotype and transient immune suppression would be required to avoid the elimination of rAAV particles by neutralizing antibodies.

Minidystrophin coded by two AAV vectors (dual vector system)

Microdystrophin proteins, with 3–4 spectrin-like repeats in the rod domain, do not completely compensate for the lack of full-length dystrophin. Among the constituents of DGC and its binding proteins, the expression of nNOS cannot be recovered through the introduction of microdystrophin.

In an attempt to introduce a therapeutic gene larger than 4.7 kb into target cells, the trans-splicing approach, in which the gene is split between two rAAV vectors, each containing part of an intron with either a splice-acceptor or a splice-donor sequence, has been developed. After formation of head-to-tail concatamers, trans-splicing of the two RNA transcripts from the two different expression cassettes removes the intervening sequence, producing a functional mRNA larger than could be delivered in a single vector. This approach was employed to deliver a minidystrophin to *mdx* muscle [20], however, the coordinated nature of transcription and splicing makes this strategy highly inefficient *in vivo*.

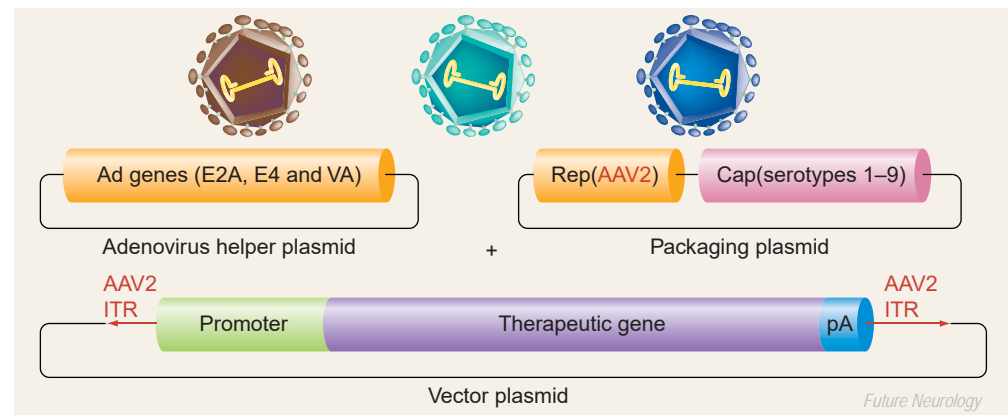
Production of AAV vectors on a large scale

rAAV vector plasmids are generated by deleting the viral genome except for the inverted terminal repeats. To obtain recombinant AAV particles, double [21] or triple [22] transfection of the plasmids into human embryonic kidney (HEK)293 cells is performed to provide rep and capsid proteins and adenoviral helper functions (Figure 3). The obtained AAV vectors are further purified by CsCl gradient sedimentation or ion-exchange chromatography. In the case of rAAV2, it is estimated that at least 1×10^{13} vg/kg is required to treat humans with hemophilia, whereas the titer of the vectors prepared by this standard method is approximately $2\text{--}5 \times 10^{13}$ genome copies from 1×10^9 HEK293 cells [13]. In clinical trials, an inexpensive, safe, large-scale system must be developed for the production of AAV. For example, Urabe and colleagues described a highly powerful production of rAAV using non-mammalian cell culture [23]. Okada and colleagues described a large-scale AAV vector production with active gassing [24].

Safety issues

Currently, most research on AAV-mediated gene transfer focuses on the systemic delivery of therapeutic genes via the blood circulation. Some have demonstrated the effectiveness of high-pressure arterial [25] or venous [26] infusion. These procedures seem to be powerful in transducing the therapeutic genes into targeted muscle groups, but the safety should be tested carefully in larger animal models. In particular, the mutagenic and carcinogenic potentials of recombinant genomes should be investigated, in addition to their potential for germline transfer after systemic delivery.

Figure 3. Production of pseudo-typed adeno-associated virus vectors in HEK293 cells.



To obtain recombinant (r)AAV, three plasmids are transfected into human embryonic kidney (HEK)293 cells. The therapeutic gene is inserted between the two ITRs of the virus, with a ubiquitous or muscle-specific promoter. To avoid contamination of the helper adenovirus, plasmids encoding adenovirus-derived genes and *rep* and *cap* genes are cotransfected into the cells. Recently, the viral capsid gene from other AAV serotypes has been preferentially used in combination with the AAV2 vector to target the musculature body-wide *in vivo* after systemic delivery or to avoid pre-existing neutralization antibodies. AAV: Adeno-associated virus; Cap: Capsid; ITR: Inverted terminal repeats.

Gutted adenoviral vectors expressing full-length dystrophin

Adenoviral vectors infect both dividing myoblasts and terminally differentiated muscle fibers, and possess a large insert capacity. However, early generations of adenoviral vectors, however, elicited substantial immune reactions in immunocompetent *mdx* mice and, hence, a rapid loss of transgene expression [27,28]. To circumvent this problem, a 'gutted' adenoviral vector, from which most viral DNA sequences are deleted, has been developed. Gutted adenoviral vectors are capable of carrying the large dystrophin gene together with regulatory sequences, and show reduced immunotoxicity compared with conventional adenoviral vectors [29-31]. Preparation of the gutted adenovirus vector requires a conventional adenovirus to supply replication and packaging functions *in trans*, and therefore has a high risk of helper virus contamination that may elicit immunological reactions upon delivery to tissues. In addition, recombinant adenoviral vectors remain comparatively toxic, especially in the liver, when administered systemically and have yet to achieve comparable transduction efficiency compared with AAV vectors.

Other vectors

A lentiviral vector is an alternative option for *in vivo* gene transfer into skeletal muscle. Kobinger and colleagues demonstrated that a lentiviral

vector encoding minidystrophin targeted both satellite cells and myofibers of *mdx* mice and provided functional correction *in vivo* [32].

Direct injection of naked plasmid into dystrophic animals

Direct injection of a naked plasmid containing a full-length dystrophin cDNA into the muscles of DMD patients has been proposed as a promising treatment to restore the expression of dystrophin. The efficiency was low in animal models [33,34] and in a Phase I gene therapy clinical trial [35], but the dystrophin expression is relatively stable and evoked no signs of humoral or cellular immune responses. Experiments using mouse models demonstrated that the efficiency of gene transfer can be enhanced by electroporation coupled with the intramuscular application of hyaluronidase [36,37]. However, combination of electroporation and hyaluronidase administration would act to damage the muscle. The application to DMD patients is questionable. Hydrodynamic delivery of naked plasmid DNA expressing full-length dystrophin into the *mdx* mice has been reported to be effective [38]. Dystrophin expression was seen in 1-5% of the myofibers of the targeted muscle group of the hind limb for an extended period. To protect dystrophin-deficient muscles from muscle degeneration, repeated administration of plasmids would be required.

Ex vivo gene transfer into myogenic cells

Cell-mediated therapy can be used to deliver the normal dystrophin gene to dystrophic muscle. In particular, *ex vivo* transfer of a functional dystrophin gene into patients' satellite cells (myogenic progenitor cells usually located between myofibers and muscle basal lamina in a dormant state) and their progeny (myoblasts) is an attractive option for cell-based therapies for DMD since several methods to freshly purify satellite cells from muscle have been established [39,40]. A lentivirus vector would be the first choice for *ex vivo* mini- or micro-dystrophin gene transfer into autologous myogenic cells because it can infect freshly isolated satellite cells without lowering their proliferation and differentiation potential [Ikemoto *et al.*, Unpublished Data]. Stem cells other than satellite cells, such as muscle side population (SP) cells [41–43], mesoangioblasts [44], and AC133-positive human stem cells [43], have been reported to participate in muscle regeneration. Muscle SP cells are isolated by their ability to efflux Hoechst dye. Bachrach and colleagues demonstrated that SP cells from *mdx* (5cv) mice transduced with microdystrophin *ex vivo* were transplanted successfully via the tail vein and delivered human microdystrophin to the skeletal muscle of nonirradiated *mdx* (5cv) mice [45]. Recently, Dezawa and colleagues reported a novel method to induce muscle progenitor cells from human bone marrow stromal cells with a high efficiency [46].

Correction of endogenous genes

Gene conversion using chimeraplasts attempts to correct point mutations of the *DMD* gene in the cell. The first generation of chimeraplasts comprises hybrid RNA/DNA molecules that are homologous to a targeted gene, yet include one mismatched base. These hybrid nucleotides trigger gene conversion from a mutant to a functional allele via intranuclear DNA mismatch repair mechanisms. Injection of chimeric oligonucleotides into *mdx* mice resulted in the expression of full-length dystrophin in muscle fibers at the site of injection [47]. Gene correction mediated by chimeraplasts has also been demonstrated in the dystrophic golden retriever dog [48]. A second generation gene editing tool is a linear DNA oligonucleotide, 25-mer or longer containing a single central mismatch. This tool repaired single point mutations in the dystrophin gene with efficiencies comparable to that seen with chimeric RNA/DNA oligonucleotides, but yielded

more consistent results [49]. Approximately 20% of DMD patients have single point mutations and, therefore, are potential targets of this therapeutic approach. However, gene repair techniques may not work for all mutations. Further data on the efficacy of the correction *in vivo* are required, using a range of point mutations of the dystrophin gene.

Targeted exon skipping

Antisense oligonucleotides

DMD and *mdx* muscles have a few revertant fibers that express functional dystrophin [50,51]. This phenomenon is explained by aberrant splicing, which omits one or more exons and, as a result, restores a disrupted reading frame and dystrophin expression. Based on this observation, forced exon skipping is being developed as a future treatment to restore dystrophin expression from the mutated DMD gene in humans. The main tools for targeted exon skipping are antisense oligonucleotides (AOs). 2'-O-methyl-modified RNA on a phosphorothioate backbone, endowed oligonucleotides with greater resistance to nuclease degradation and, therefore, additional increases in stability were achieved [52–54]. Direct intramuscular injection of 2'-O-methyl phosphorothioate AOs resulted in a significant increase in the number of dystrophin-positive fibers (20%) in *mdx* mouse muscle [55]. Phosphoro-amide morpholino oligonucleotides have also proven to be effective in producing functional dystrophin in dystrophin-deficient muscle [56]. Weekly intravenous injections of morpholino AOs induced the expression of functional levels of dystrophin body-wide in skeletal muscles of the dystrophic *mdx* mouse and improved muscle function [57]. Based on the successful results in animal models, a clinical trial using AOs has already started in Leiden and is about to commence in the UK. Theoretically, AO-based exon skipping is applicable to 80% of dystrophin gene mutations. Furthermore, it is estimated that targeting just 12 exons restores the open reading frame of 75% of all deletions responsible for DMD.

AAV-mediated exon skipping

AOs display a limited half-life *in vivo*, and administration of AOs to patients must be repeated weekly or monthly. To obtain a longer-term effect, rAAV1 vectors expressing a modified U7 small nuclear RNA gene were used to direct exon skipping in *mdx* mice [58]. Following a single, high-pressure injection of the rAAV1/U7 vector

into the femoral artery of mdx mice, normal levels of dystrophin expression were restored and sustained for over 6 months. Although the initial study was limited to delivery to a single limb, this technique could be coupled with systemic delivery of AAV vectors of new serotypes.

Insulin-like growth factor-1 & myostatin blockade rescue dystrophin-deficient muscle

Myostatin (also known as growth and differentiation factor [GDF]8) is a transforming growth factor (TGF)- β family member that negatively regulates skeletal muscle growth, as evidenced by the increased musculature of the mice with a null mutation in this gene [59]. Mutation of the myostatin gene has also been found in human [60]. The myostatin-null child was reported to be muscular without any health problems at 4.5 years of age [60]. Myostatin blockade in *mdx* mice results in increases in both muscle mass and muscle strength and reductions in muscle fiber degeneration and serum creatine kinase levels [61]. Based on this observation, the recombinant human antibody against myostatin (MYO-029) is now being tested on adult muscular dystrophy patients.

Increased insulin-like growth factor (IGF)-1 within *mdx* myofibers reduces the breakdown of dystrophic muscle during the acute onset of muscle degeneration [62]. This mechanism of action can partly account for the long-term reduced severity of the dystrophic pathology in *mdx* mice over-expressing mIGF-1 and provides opportunities for therapeutic strategies [63].

Conclusion

Almost 20 years have passed since the discovery of dystrophin. Unfortunately, we have yet to find an effective therapy that can mitigate the dystrophic process. Numerous approaches are currently being explored, but many suffer from a variety of drawbacks. Among the gene therapy approaches to DMD under investigation, rAAV-mediated gene transfer is the most

promising but still faces several obstacles. Other therapeutic approaches, including cell therapy and pharmacological intervention, would be used in complement with AAV-microdystrophin gene transfer.

Future perspective

An important step towards the clinical use of gene therapy is the evaluation of the efficacy and safety of gene transfer methods and protocols using animals larger than mice. We have established a beagle-based canine X-linked muscular dystrophy (CXMD) colony at the National Institute of Neuroscience in Japan (CXMD_J) and reported their severe phenotypes [64]. Beagle-based CXMD_J is smaller and easier to handle than golden retriever CXMD, and is, therefore, a useful model for DMD. Preclinical studies using nonhuman primates would also be informative before clinical trials. Importantly, there are so many variables, even in a single treatment, such as myostatin blockade with antibodies, that more trials will be needed.

At present, gene therapy trials and related strategies face various hurdles and difficulties. Effective treatment of DMD may be achieved through a combination of different therapeutic approaches; for example, a combination of AAV vector-mediated gene transfer plus corticosteroid administration or myostatin blockage.

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Executive summary

Introduction

- Duchenne muscular dystrophy (DMD) is caused by mutations in the *DMD* gene, which encodes a 427-kDa subsarcolemmal cytoskeletal protein, dystrophin.
- At present, there is no treatment to arrest the progression of DMD and patients generally suffer from respiratory and/or cardiac complications in the second decade of life.
- Among several therapeutic strategies for this disease, recombinant adeno-associated virus (rAAV)-mediated gene transfer is the most promising.

Executive summary

Viral vector-mediated gene therapy

- AAV vectors drive long-term expression of the therapeutic gene in skeletal muscle *in vivo*, but the insertion size is limited to 4.9 kb.
- Functional, rod domain-deleted dystrophin (microdystrophin) can be incorporated into AAV vectors.
- New serotypes of AAV vectors have been isolated and developed as gene-transfer vectors, some of which transport the therapeutic genes to all the muscles of the body after systemic delivery.

Ex vivo gene transfer into myogenic stem cells

- Cell-mediated therapy can be used to deliver a normal dystrophin gene to dystrophic muscle in the hope that the delivered cells will participate in muscle-fiber regeneration in dystrophic muscle, express dystrophin and improve muscle function.
- Muscle satellite cells, side population cells, mesangioblasts, AC133-positive cells and bone marrow stromal cells are expected to be potential cell sources for cell-mediated therapy.

Gene correction & exon skipping using antisense oligonucleotides

- Chimeraplasts, which are chimeric RNA/DNA oligonucleotides homologous to a targeted gene (except for the inclusion of one mismatched base) can be used to direct the correction of a mutation by inducing preferential gene conversion from a mutant to a functional allele.
- Exon skipping using antisense oligonucleotides (AOs) targets transcribed RNA molecules to omit a nonsense mutation and restore a disrupted reading frame.
- Weekly intravenous injections of morpholino phosphorodiamidate (morpholino) AOs induce the expression of functional levels of dystrophin in skeletal muscles body-wide in the dystrophic *mdx* mouse.

Myostatin & insulin-like growth factor-1

- Blockage of myostatin and delivery of insulin-like growth factor-1 are effective to improve dystrophic phenotypes and the contractile force of dystrophin-deficient muscle.

Future perspective

- Preclinical studies using dystrophic dogs and nonhuman primates would be informative before human clinical trials.
- To overcome this devastating disease, multiple, diverse therapeutic strategies should be combined.

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